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(19)(CA) **APPLICATION FOR CANADIAN PATENT** (12)

(54) Methods for Treatment or Diagnosis of Diseases or Conditions Associated with Abnormal Signal Transduction

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2187781

1

METHODS FOR TREATMENT OR DIAGNOSIS OF DISEASES OR CONDITIONS ASSOCIATED WITH ABNORMAL SIGNAL TRANSDUCTION

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Field of the Invention

The present invention relates generally to the field of cellular signal transduction and more specifically to the diagnosis and treatment of various diseases and conditions associated with abnormal cell proliferation.

Background of the Invention

The following is a discussion of relevant art, none of which is admitted to be prior art to the invention.

Cellular signal transduction is a fundamental mechanism whereby external stimuli that regulate diverse cellular processes are relayed to the interior of cells. One of the key biochemical mechanisms of signal transduction involves the reversible phosphorylation of tyrosine residues on proteins. The phosphorylation state of a protein is modified through the reciprocal actions of tyrosine kinases (TKs) and tyrosine phosphatases (TPs).

Receptor tyrosine kinases belong to a family of transmembrane proteins and have been implicated in cellular signaling pathways. The predominant biological activity of some receptor tyrosine kinases is the stimulation of cell growth and proliferation, while other receptor tyrosine kinases are involved in arresting growth and promoting differentiation. In some instances, a single tyrosine kinase can inhibit, or stimulate, cell proliferation depending on the cellular environment in which it is

expressed. (Schlessinger, J. and Ullrich, A., *Neuron,*
9(3):383-391, 1992.)

Receptor tyrosine kinases are composed of at
least three domains: an extracellular ligand binding
5 domain, a transmembrane domain and a cytoplasmic catalytic
domain that can phosphorylate tyrosine residues. Ligand
binding to membrane-bound receptors induces the formation
of receptor dimers and allosteric changes that activate
the intracellular kinase domains and result in the self-
10 phosphorylation (autophosphorylation and/or transphospho-
rylation) of the receptor on tyrosine residues. Indivi-
dual phosphotyrosine residues of the cytoplasmic domains
of receptors may serve as specific binding sites that
interact with a host of cytoplasmic signalling molecules,
15 thereby activating various signal transduction pathways
(Ullrich, A. and Schlessinger, J., 1990, Cell 61:203-212).

The intracellular, cytoplasmic, non-receptor
protein tyrosine kinases do not contain a hydrophobic
transmembrane domain and share non-catalytic domains in
20 addition to sharing their catalytic kinase domains. Such
non-catalytic domains include the SH2 domains (SRC
homology domain 2; Sadowski, I. et al., Mol. Cell. Biol.
6:4396-4408; Koch, C.A. et al., 1991, Science 252:668-674)
and SH3 domains (SRC homology domain 3; Mayer, B.J.
25 et al., 1988, Nature 332:269-272). Such non-catalytic
domaims are also thought to include the PH domain
(Musacchio et al., 1993, TIBS 18:342-348). The non-
catalytic domains are thought to be important in the
regulation of protein-protein interactions during signal
30 transduction (Pawson, T. and Gish, G., 1992, Cell 71:359-
362).

A central feature of signal transduction (for
reviews, see Posada, J. and Cooper, J.A., 1992, Mol. Biol.
Cell 3:583-392; Hardie, D.G., 1990, Symp. Soc. Exp. Biol.
35 44:241-255), is the reversible phosphorylation of certain
proteins. Receptor phosphorylation stimulates a physical
association of the activated receptor with target mole-

3

cules.　Some of the target molecules are in turn phospho-
rylated.　Such phosphorylation transmits a signal to the
cytoplasm.　Other target molecules are not phosphorylated,
but assist in signal transmission by acting as adapter
5　molecules for secondary signal transducer proteins.　For
example,　receptor phosphorylation and the subsequent
allosteric changes in the receptor recruit the *Grb-2/SOS*
complex to the catalytic domain of the receptor where its
proximity to the membrane allows it to activate *ras*
10　Pawson, T. and Schlessinger, J., *Current Biol.* 13:434,
1993.

The　secondary　signal　transducer　molecules
generated by activated receptors result in a signal
cascade that regulates cell functions such as cell
15　division　or　differentiation.　Reviews　describing
intracellular signal transduction include Aaronson, S.A.,
Science, *254*:1146-1153, 1991;　Schlessinger, J. *Trends
Biochem. Sci.*, *13*:443-447, 1988; and Ullrich, A., and
Schlessinger, J., *Cell*, *61*:203-212, 1990.
20　Dimerization of cell-surface receptors upon bind-
ing of their cognate ligand appears to be a primary event
in transmembrane signaling (Ullrich and Schlessinger, *Cell*
61:203-212, 1990; Canals, *Biochemistry* 31:4493-4501,
1992).　Studies indicate that dimerization is sufficient
25　for receptor activation (Schreiber et al., *Proc. Natl.
Acad. Sci. USA* 78:7535-7539, 1981;　Sorokin et al.,
J. Biol. Chem. 269:9752-9759, 1994; Watowich et al., *Proc.
Natl. Acad. Sci. USA* 89:2140-2144, 1992), and propose that
ligand-induced receptor dimerization constitutes a general
30　mechanism for transmembrane signaling by these receptors
(Schlessinger and Ullrich, *Neuron* 9:383-391, 1992).

Ligand-induced receptor dimerization is thought
to occur via two possible mechanisms, or a combination of
them.　In the first, binding of the ligand to its cognate
35　receptor may be bivalent, as is seen in the case of
dimeric ligands such as PDGF, CSF1 and SCF.　Binding of
each protomer of the ligand dimer to a separate receptor

2187781

4

molecule results in cross-linking and subsequent activation of the receptor (Heldin et al., *J. Biol. Chem.* 264:8905-8912, 1989; Lev et al., *J. Biol. Chem.* 267:15970-15977, 1992). Human growth hormone (hGH) also binds
5   bivalently to its receptor, despite being monomeric in solution. A number of biophysical methods (Cunningham et al., *Science* 254:821-825, 1991), including X-ray crystallography (de Vos et al., *Science* 255:306-312, 1992), have shown that a single molecule of hGH binds
10 simultaneously to two receptor molecules, each of which has a single site for hGH binding. This results in dimerization of the receptor, augmented by extensive interactions between the receptor molecules themselves, resulting in its activation.
15   The other proposed mechanism involves ligand-induced conformational changes in the receptor molecule that result in stabilization of its dimeric form. This mechanism is most likely when a single monomeric ligand molecule binds to each receptor. Studies of EGF binding
20 to the extracellular domain of its receptor, using circular dichroism (CD) and fluorescence spectroscopy, have shown that conformational changes occur upon ligand binding (Greenfield et al., *EMBO J.* 8:4115-4123, 1989), and it has been proposed that these may be responsible for
25 EGF-induced dimerization and activation of its receptor.
        In the case of receptors that pre-exist in a dimeric form, such as the insulin receptor and bacterial chemotactic receptors, signal transduction is also proposed to occur through ligand-induced conformational
30 changes. The aspartate receptor (Tar) of *S. typhimurium* has two binding sites for aspartate per dimer, which show negative cooperativity (Biemann and Koshland, *Biochemistry* 33:629-634, 1994). Binding of aspartate to one of these sites results in an altered juxtaposition of the two
35 protomers of the dimer (Milburn et al., *Science* 254:1342-1347, 1991; Yeh et al., *J. Biol. Chem.* 268:9787-9792, 1993). Ligand-binding thus induces a

2187781

5

transition between two particular dimeric states of the receptor, one of which is active. This can be considered as formally equivalent to a ligand-induced transition from a monomeric to a dimeric state, and shows many
5 similarities with the activation of hGH-R.

      The insulin receptor pre-exists as a dimer, with each protomer consisting of an α- and a β-chain. CD and fluorescence spectroscopic studies have shown that insulin binding is associated with substantial conformational
10 changes in the extracellular domain of the receptor (Schaefer et al., *J. Biol. Chem.* 267:23393-23402, 1992).

      It has previously been shown that EGF binds to the soluble extracellular domain of its receptor (sEGFR), and stimulates its dimerization (Lax et al., *J. Biol.*
15 *Chem.* 266:13828-13833, 1991; Hurwitz et al., *J. Biol. Chem.* 266:22035-22043, 1991). The cross-linked dimeric form of sEGFR has been reported to have a higher affinity for EGF than the monomeric form (Zhou et al., *Biochemistry* 32:8193-8198, 1993). EGF does not appear to bind to
20 accessory molecules. It must induce dimerization of its receptor by inducing conformational changes (Greenfield et al., *EMBO J.* 8:4115-4123, 1989) that stabilize the dimeric form, and/or by binding to the receptor in a multivalent manner, as observed for hGH (Cunningham et al., *Science*
25 254:821-825, 1991). Numerous attempts to crystallize the EGF/sEGFR complex for X-ray crystallographic analysis have so far been unsuccessful (Günther et al., *J. Biol. Chem.* 265:22082-22085, 1990), so a detailed view of ligand-induced conformational changes and dimerization is
30 not available.

      Abnormalities in signal transduction pathways can lead to various diseases in at least three different ways: (1) underactivity (2) mutation, and (3) overexpression. An example of underactivity is observed in some forms of
35 diabetes. Examples of mutation include the role of BCR-ABL in chronic myelogenous leukemia and acute lymphocytic leukemia. Pendergrast et al., 1993, Cell 75:175-185.

6

Overexpression of certain protein tyrosine kinases has been shown to subvert normal growth control pathways and lead to oncogenesis (reviewed in Hunter, T., 1991, Cell 64:249-270). An example of a protein that may

5   participate in the aberrant growth of breast cancer cells is HER2, also known as c-erbB-2 (Coussens et al., 1985 Science 230:1132-1139; Yamamoto et al., 1986, Nature, 319:521-527). This receptor was also isolated as the rat oncogene neu, an oncogene responsible for chemically

10 induced rat glioblastomas (Padhy et al., 1982 Cell, 28:865-871; Schechter et al., 1984 Nature 312:513-516; Bargmann et al., 1986, Nature, 319:226-230). HER2/erbB-2 is known to be amplified and overexpressed in about 25% of human breast cancers (Slamon et al., 1987 Science 235:177-

15 182; Slamon et al., 1989 Science 244:707-712).

Various cell proliferative disorders have been associated with defects in different signaling pathways mediated by receptor tyrosine kinases. According to Aaronson, *Science*, 254:1146-1153, 1991:

20   Signaling pathways that mediate the
              normal functions of growth factors are
              commonly subverted in cancer.

Examples of specific receptor tyrosine kinases associated with cell proliferative disorders include,

25 platelet derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), and HER2. The gene encoding HER2 (her-2) is also referred to as *neu*, and *c-erbB*-2 (Slamon, D.J., et al., *Science*, 235:177-182, 1987). HER2/*neu* gene amplification has been linked by some

30 investigators to neoplastic transformation.

Publications discussing EGFR and cancer include Zeillinger et al., Clin. Biochem. 26:221-227, 1993; where it is asserted:

              Increased expression of this receptor
35   [EGFR] has been found in various malig-
              nancies. In carcinomas of the cervix,
              ovaries, esophagus, and stomach,
              positive EGF-R status is definitely
              associated with the aggressiveness of
40   the tumor.

7

With regard to breast cancer the import-
ance attached to the determination of
EGF-R has been confirmed by reports by
several   groups   on   the   positive
5   correlation between EGF-R and relapse-
free interval, as well as overall
survival. [Citations omitted.]

Other references discussing cancer and EGFR
include Karameris et al., Path. Res. Pract. 189:133-137,
10   1993; Hale et al., J. Clin. Pathol. 46:149-153, 1993;
Caraglia et al., Cancer Immunol. Immunother. 37:150-156,
1993; and Koenders et al., Breast Cancer Research and
Treatment 25:21-27, 1993).

Some small molecules and antibodies have been
15   studied for their ability to affect biological activities
associated with receptors of the EGF family.   Some
compounds that have been studied for their ability to
block EGF-dependant cell proliferation have been said to
block EGF-receptor autophosphorylation less effectively
20   than exogenous-substrate phosphorylation (Yaish et al.,
Science 242:933-935, 1988). The drug leflunomide has been
reported to inhibit cellular proliferation by inhibiting
tyrosine-specific kinase activity (Mattar et al., FEBS,
334(2):161-164, 1993).   Other small molecules, termed
25   tyrphostins, have been studied for their ability to
selectively inhibit either HER1 or HER2 in vitro (Osberov
et al., J. Biol. Chemistry 268:11134-11142, 1993). One
study that was performed using antibodies has reported
that blockade of the EGF receptor inhibits TGF-alpha
30   induced, but not estrogen induced, hormone-dependant human
breast cancer.   Arteaga et al., Mol. Endocrinology,
2:1064-1070, 1988.

Compounds such as tryphostin and leflunomide act
intracellularly to block the interaction between the
35   kinase and its substrate rather than by blocking the
dimerization or activation of the receptor, and thus must
be able to traverse the cell membrane. The cyano group of
the tryphostins may be toxic, especially if two cyano
groups are present.

8

Summary of the Invention

This invention relates to methods for treatment
of a disease or condition in organisms, or for diagnosis
of such diseases or conditions involved in abnormal
5   protein-protein interactions.

These interactions are associated with the basic
signalling function of proteins associated with various
diseases or conditions. Such diseases or conditions
include cell adhesion diseases, neuronal diseases,
10. oncogenic disorders, diseases associated with defects in
cellular movement or in developmental processes. For
example, specific diseases or conditions include breast
cancer, atherosclerosis, inflammation, and diseases
associated with inappropriate activities of platelets,
15   mononuclear, or neutrophil cells.

Measurement of the level of interaction has been
determined to be a useful measure of the existence of
certain diseases or conditions. The specific interactions
encompassed by the present invention include the
20   interaction between a BLM domain and its natural binding
partner and the interaction between domain 1 and/or 3 of
the EGF receptor and an EGF ligand. Such diseases or
conditions are thus characterized by inappropriate
interaction. Diseases or conditions characterized by
25   abnormal levels of interaction can be alleviated to some
extent by disrupting or promoting that interaction
in vivo. In addition, useful agents for treatment of such
diseases can be identified by standard screening protocols
in which measurement of such interaction is determined.
30   For example, such an agent may be a peptide which either
comprises, consists of, or consists essentially of the BLM
domain, domain 1 or 3 of thr EGF receptor, or a ligand
that binds any of the above domains.

This invention also relates to methods for
35   diagnosis and treatment of a disorder, most preferably a
disorder characterized by an abnormality in a signal
transduction pathway, wherein the signal transduction

9

pathway involves one of the interactions mentioned above.
The present invention is based in part on the discovery of
a new domain, herein termed the BLM domain, and also in
part on the determination that simultaneous binding of a
5 single molecule of EGF to two different regions on two
receptor molecules causes ligand-induced stabilization of
the active dimer of the receptors.

Thus, in a first aspect the invention features a
method for treatment of a disease or condition in an
10 organism characterized by an abnormal level of interaction
between: (a) a BLM domain and its natural binding partner;
or (b) domain 1 and/or 3 of an EGF receptor and an EGF
ligand. The disease or condition may also be
characterized by an abnormality in a signal transduction
15 pathway, wherein the pathway contains a protein with a
domain listed above. The method includes disrupting or
promoting that interaction (or signal) _in vivo_. The
method also involves inhibiting the activity of the
complex formed between the components listed above.

20 By "disease or condition" is meant a state in an
organism, _e.g._, a human, which is recognized as abnormal
by members of the medical community. The disease or
condition may be characterized by an abnormality in one or
more signal transduction pathways in a cell, preferably an
25 epidermal or cancer cell, wherein one of the components of
the signal transduction pathway is an EGF receptor or a
protein having a BLM domain. The disease or condition may
also be characterized by abnormal cell proliferation.
Examples of diseases or conditions to be treated or
30 diagnosed by the present invention include various cancers
and cell proliferative disorders. Particular cancers that
can be diagnosed and treated by the present invention
include leukemia, epithelial carcinomas, cancers of the
breast, bladder, lung, stomach and ovaries, and a variety
35 of adenocarcinomas. These and other diseases or
conditions are often characterized by one or more symptoms
such as tumors.

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"Cell proliferative disorders" refer to disorders
wherein unwanted cell proliferation of one or more
subset(s) of cells in a multicellular organism occurs,
resulting in harm (e.g., discomfort or decreased life
5   expectancy) to the multicellular organism. Cell
proliferative disorders can occur in different types of
animals and in humans. Cell proliferative disorders
include cancers and psoriasis. A particular disorder is
considered to be "driven" or caused by a particular
10   receptor tyrosine kinase if the disorder is characterized
by over-activity, or inappropriate activity, of the kinase
and a compound which can inhibit the kinase activity
exerts a therapeutic effect when administered to a patient
having the disorder.

15   By "organism" is meant any living creature. The
term includes mammals, and specifically humans. Preferred
organisms include mice, as the ability to treat or
diagnose mice is often predictive of the ability to func-
tion in other organisms such as humans.

20   By "abnormality" is meant an a level which is
statistically different from the level observed in organ-
isms not suffering from such a disease or condition and
may be characterized as either an excess amount, intensity
or duration of signal, or a deficient amount, intensity or
25   duration of signal.   The abnormality in signal
transduction may be realized as an abnormality in cell
function, viability or differentiation state. We have
determined that such abnormal interaction in a pathway can
be alleviated by action at the EGF receptor-EGF ligand
30   interaction site in the pathway or at the BLM domain-
natural binding partner site. An abnormal interaction
level may also either be greater or less than the normal
level and may impair the normal performance or function of
the organism. Thus, it is also possible to screen for
35   agents that will be useful for treating a disease or
condition, characterized by an abnormality in the signal
transduction pathway, by testing compounds for their

2187781

11

      ability to affect the interaction of interest, since the
complex formed by such interaction is part of the signal
transduction pathway. However, the disease or condition
may be characterized by an abnormality in the signal
5 transduction pathway even if the level of interaction
between the components of interest is normal.

      By "signal transduction pathway" is meant the
sequence of events that involves the transmission of a
message from an extracellular protein to the cytoplasm
10 through a cell membrane. The signal ultimately will cause
the cell to perform a particular function, for example, to
proliferate and therefore cause cancer. Various mechan-
isms for the signal transduction pathway (Fry et al.,
1993, Protein Science, 2:1785-1797) provide possible
15 methods for measuring the amount or intensity of a given
signal. Depending upon the particular disease associated
with the abnormality in a signal transduction pathway,
various symptoms may be detected. For example, if the
disease is breast cancer, one may detect cell prolifera-
20 tion or tumor size, among other symptoms. Those skilled
in the art recognize those symptoms that are associated
with the various other diseases described herein.
Furthermore, since some adaptor molecules recruit
secondary signal transducer proteins towards the membrane,
25 one measure of signal transduction is the concentration
and localization of various proteins and complexes. In
addition, conformational changes that are involved in the
transmission of a signal may be observed using circular
dichroism and fluorescence studies. One signal
30 transduction pathway that has been studied is the Ras
signalling pathway. (Pendergrast et al., 1993, Cell
75:175-185) The role of the GRB2 protein in the Ras
signalling pathway (and the interaction with BCR-ABL, an
oncoprotein implicated in certain human leukemias) was
35 elucidated using a combination of biochemical and genetic
studies. Similar studies are expected to elucidate the
specific signalling pathway(s) affected by BLM domain

12 -

containing proteins such as Grb7 or EGF receptor domain 1
or 3 containing proteins.

By "interact" is meant any physical association
between proteins, whether covalent or non-covalent.

5　Examples of non-covalent bonds include electrostatic
bonds, hydrogen bonds, and Van der Waals bonds. Stryer,
Biochemistry, 1988, pages 7-8. Furthermore, the interac-
tions between proteins may either be direct or indirect.
Thus, the association between two given proteins may be

10　achieved with an intermediary agent, or several such
agents, that connects the two proteins of interest.
Another example of an indirect interaction is the indepen-
dent production, stimulation, or inhibition of both an EGF
receptor domain (i.e., domain 1 and/or domain 3) or BLM

15　domain and EGF ligand or BLM domain natural binding
partner by a regulatory agent (i.e., a gene transfer
vehicle containing DNA encoding domain 1 and/or domain 3
of the EGF receptor or the BLM domain). Depending upon
the type of interaction present, various methods may be

20　used to measure the level of interaction. For example,
the strengths of covalent bonds are often measured in
terms of the energy required to break a certain number of
bonds (i.e., kcal/mol) Non-covalent interactions are
often described as above, and also in terms of the

25　distance between the interacting molecules. Indirect
interactions may be described in a number of ways,
including the number of intermediary agents involved, or
the degree of regulatory control exercised over one domain
relative to the regulatory control exercised over another

30　domain to which the first domain binds.

The term "BLM domain" is defined below.

By "natural binding partner" is meant a protein
that interacts with a BLM domain. Given that the PH
domain is a subdomain of the present BLM domain, it is

35　possible that some or all of the natural binding partners
for the PH domain may also serve as natural binding part-
ners for the BLM domain. In addition, a natural binding

13

partner for a BLM domain may only interact with the BLM
domain and not the PH domain. The structure (primary,
secondary, or tertiary) of the particular natural binding
partner will influence the particular type of interaction
5 between the BLM domain and the natural binding partner.
For example, if the natural binding partner comprises a
sequence of amino acids complementary to the BLM domain,
covalent bonding may be a possible interaction.
Similarly, other structural characteristics may allow for
10 other corresponding interactions. The interaction is not
limited to particular residues and specifically may
involve phosphotyrosine, phosphoserine, or
phosphothreonine residues. A broad range of sequences may
be capable of interacting with BLM domain. Experience
15 with another signalling domain, the SH2 domain, has
demonstrated that the range of sequences that binds that
given domain may be much broader than was initially
expected. Fry et al, Protein Science, 1993, 2:1785-1797.
Therefore, using techniques well known in the art, one may
20 identify several natural binding partners for the BLM
domain.

By "EGF receptor" is meant a receptor that
interacts with an EGF ligand through domains 1 and/or 3.
An EGF receptor may be any member of the family or group
25 of RTK's that is frequently overexpressed in a variety of
epithelial carcinomas, such as EGFR, HER2, HER3 and HER4.
(Plowman et al., Proc. Natl. Acad. Sci. USA 90: 1746-1750,
1993, incorporated herein by reference.) An EGF receptor
typically has a hydrophobic transmembrane region that
30 divides an extracellular ligand binding domain and a C-
terminal cytoplasmic domain, both of which can be further
be divided into four subdomains. Those skilled in the art
can recognize other EGF receptors using techniques well-
known in the art such as sequence comparison (i.e.,
35 looking for sequences with a similar length to the
examples listed above and at least 34% identical to the
extracellular domains, especially at the approximately 50

14

conserved cysteine residues; and at least 19% identical to
the cytoplasmic domains). See, Plowman, supra. Other
references that may be relevant to describing EGF
receptors include Lax et al., EMBO 8:421-427, 1989; Avivi
5   et al., Oncogene 6:673-673, 1991; Lax et al., Cell
Regulation 2:337-345, 1991; and Lax et al., Molecular and
Celluar Biology 8:1970-1978, 1988, all of which are hereby
incorporated herein by reference. EGF receptors are found
in a variety of animals, including humans, chickens and
10   mice. See, Avivi, supra.

By "EGF ligand" is meant a molecule, preferably
a polypeptide, that binds to an EGF receptor, preferably
at domain 1 and/or 3. Non-limiting examples of EGF
ligands include EGF, transforming growth factor α,
15   amphiregulin, heparin-binding EGF, vaccinia virus growth
factor, gp30, and heregulin. Those skilled in the art can
identify other EGF ligands using procedures well-known in
the art.

Domains 1 and 3 of an EGF receptor are bound
20   simultaneously by a single EGF ligand and are involved in
EGF receptor dimerization and corresponding signal
transduction and cell proliferation. By "domain 1" is
meant a subdomain found in EGF receptors that binds EGF
ligands. Domain 1 is typically flanking rather than
25   cysteine-rich and generally corresponds approximately to
residues 25-163 of EGFR or 29-185 of HER4. By "domain 3"
is meant a subdomain found in EGF receptors that binds EGF
ligands. Domain 3 is typically flanking, rather than
cysteine-rich, and generally corresponds approximately
30   residues 313-474 of EGFR or 335-495 of HER4. Domains 1
and 3 may include noncontiguous regions of an EGF receptor
that is capable of binding to an EGF ligand. See, Lax,
1991, supra.

By "disrupt" is meant that the interaction is
35   reduced either by preventing expression of a protein con-
taining a BLM or EGF domain, or by preventing expression
of its natural binding partner, or by specifically

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preventing interaction of the naturally synthesized
proteins or by interfering with the interaction of the
proteins.

     By "promote" is meant that the interaction is
5 increased either by increasing expression of a protein
containing a BLM or EGF domain, or by increasing
expression of its natural binding partner or ligand, or by
promoting interaction of the proteins.

     In preferred embodiments, the method includes
10 providing a molecule which blocks or promotes binding of
said EGF ligand with domain 1 and/or domain 3 of the EGF
receptor and disrupts or promotes EGF receptor
dimerization (or signal) in vivo or in vitro. In
especially preferred embodiments the molecule is not an
15 antibody, has a molecular weight below 10,000 and/or is a
peptide that consists essentially of domain 1 or domain 3
or an EGF ligand fragment which binds one or both of these
domains.

     In a related aspect the invention features a
20 method for diagnosis of such a disease or condition by
detecting the level of such interaction as an indication
of that disease or condition. The diagnosis may also
involve the detection of an abnormal amount or intensity
of a signal generated by a signal transduction pathway,
25 wherein the signal transduction pathway contains a protein
having a BLM domain or a domain 1 and/or domain 3 of an
EGF receptor.

     By "diagnosis" is meant any method of identifying
a symptom normally associated with a given disease or con-
30 dition. Thus, an initial diagnosis may be conclusively
established as correct by the use of additional confirma-
tory evidence such as the presence of other symptoms.
Current classification of various diseases and conditions
is constantly changing as more is learned about the mech-
35 anisms causing the diseases or conditions. Thus, the
detection of an important symptom, such as the detection
of an abnormal level of interaction (or the detection of

16

an abnormal level of signal transduction in a pathway
containing the interaction of interest) may form the basis
to define and diagnose a newly named disease or condition.
For example, conventional cancers are classified according
5   to the location of the tumor, i.e, breast cancer, ovarian
cancer, etc. However, a subset of these cancers are both
associated with the overexpression of Her2, and as the
role of Her2 becomes more fully understood, these cancers
may be reclassified as Her2 cancers regardless of the
10   location of the tumor.

        In yet another related aspect the invention
features a method for screening for an agent useful for
treatment of such a disease or condition by assaying
potential agents for the ability to disrupt or promote
15   that interaction. The screening may also involve assaying
potential agents for the ability to remove or reduce an
abnormality in a signal transduction pathway, wherein the
signal transduction pathway contains a protein with a BLM
domain or domain 1 and/or domain 3 of an EGF receptor.

20   By "screening" is meant investigating organisms
for the presence or absence of a property. The process
includes measuring and detecting various properties,
including the level of signal transduction and the level
of interaction between the proteins and/or domains of
25   interest that are listed above. Depending upon the type
of interaction present, various methods may be used to
measure the level of interaction. For example, the
strengths of covalent bonds are often measured in terms of
the energy required to break a certain number of bonds
30   (i.e., kcal/mol) Non-covalent interactions are often
described as above and also in terms of the distance
between the interacting molecules. Indirect interactions
may be described in a number of ways, including the number
of intermediary agents involved, or the degree of control
35   exercised over one domain relative to the control
exercised over a different domain that binds the first
domain.

21877⁄81

17

The invention also features a peptide comprising,
consisting or consisting essentially of a BLM domain,
domain 1 or domain 3 of an EGF receptor or a domain
capable of inding any of the above domains.   By
5 "comprising" is meant including, but not limited to, what-
ever follows the word "comprising". Thus, use of the term
"comprising" indicates that the listed elements are
required or mandatory, but that other elements are
optional and may or may not be present. By "consisting
10 of" is meant including, and limited to, whatever follows
the phrase "consisting of". Thus, the phrase "consisting
of" indicates that the listed elements are required or
mandatory, and that no other elements may be present. By
"consisting essentially of" is meant including any
15 elements listed after the phrase, and limited to other
elements that do not interfere with or contribute to the
activity or action specified in the disclosure for the
listed elements. Thus, the phrase "consisting essentially
of" indicates that the listed elements are required or
20 mandatory, but that other elements are optional and may or
may not be present depending upon whether or not they
affect the activity or action of the listed elements.

        In preferred embodiments the peptide, protein, or
polypeptide is isolated, purified, or enriched.

25   By "isolated" in reference to a polypeptide is
meant a polymer of 2 (preferably 7, more preferably 13,
most prefereably 25) or more amino acids conjugated to
each other, including polypeptides that are isolated from
a natural source or that are synthesized. The isolated
30 polypeptides of the present invention are unique in the
sense that they are not found in a pure or separated state
in nature. Use of the term "isolated" indicates that a
naturally occurring sequence has been removed from its
normal cellular environment. Thus, the sequence may be in
35 a cell-free solution or placed in a different cellular
environment. The term does not imply that the sequence is
the only amino acid chain present, but that it is

18

essentially free (about 90 - 95% pure at least) of
non-amino acid material naturally associated with it.

By the use of the term "enriched" in reference to
a polypeptide is meant that the specific amino acid
5   sequence constitutes a significantly higher fraction (2 -
5 fold) of the total of amino acids present in the cells
or solution of interest than in normal or diseased cells
or in the cells from which the sequence was taken. This
could be caused by a person by preferential reduction in
10   the amount of other amino acids present, or by a
preferential increase in the amount of the specific amino
acid sequence of interest, or by a combination of the two.
However, it should be noted that enriched does not imply
that there are no other amino acid sequences present, just
15   that the relative amount of the sequence of interest has
been significantly increased. The term significant here
is used to indicate that the level of increase is useful
to the person making such an increase, and generally means
an increase relative to other amino acids of about at
20   least 2 fold, more preferably at least 5 to 10 fold or
even more. The term also does not imply that there is no
amino acid from other sources. The other source amino
acid may, for example, comprise amino acid encoded by a
yeast or bacterial genome, or a cloning vector such as
25   pUC19. The term is meant to cover only those situations
in which man has intervened to elevate the proportion of
the desired nucleic acid.

It is also advantageous for some purposes that an
amino acid sequence be in purified form. The term
30   "purified" in reference to a polypeptide does not require
absolute purity (such as a homogeneous preparation);
instead, it represents an indication that the sequence is
relatively purer than in the natural environment (compared
to the natural level this level should be at least 2-5
35   fold greater, e.g., in terms of mg/ml). Purification of
at least one order of magnitude, preferably two or three
orders, and more preferably four or five orders of

21B77B1

19

magnitude is expressly contemplated. The substance is
preferably free of .contamination at a functionally
significant level, for example 90%, 95%, or 99% pure.

       In preferred embodiments, the disease or condi-
5 tion which is diagnosed or treated are those described
herein, and the agent is a dominant negative mutant
protein provided by gene therapy or other equivalent
methods as described below. That is, the agent is a
peptide which blocks interaction of the BLM domain with
10 its natural binding partner or the interaction of the EGF
domain with the EGF ligand. The peptide may be
recombinant, purified, or placed in a pharmaceutically
acceptable carrier or diluent. The peptide may be used to
produce antibodies and may either contain or be
15 complementary to at least 20 amino acids from the BLM or
EGF domain. In the screening method, transformed animals,
e.g., mice containing proteins having a BLM or EGF domain,
for example, the GRB-7 or GRB-10 protein, or the F10E9.6
protein (see below) may be used.

20   By "dominant negative mutant protein" is meant a
mutant protein that interferes with the normal signal
transduction pathway. The dominant negative mutant
protein contains the domain of interest (e.g., the BLM
domain), but has a mutation preventing proper signalling,
25 for example by preventing binding of a second domain from
the same protein. One example of a dominant negative
protein is described in Millaur, B. et al., Nature
February 10, 1994. In yet other preferred embodiments,
the specific region is that corresponding to the BLM
30 domain in GRB-7 (as shown in the figures) between bases
encoding amino acids 95 to 428 and, in particular, those
bases 5' of the pleckstrin domain, i.e., between bases
encoding amino acids 95 and 231.

       Useful agents for treatment of such diseases can
35 be identified by standard screening protocols in which
measurement of such interaction is determined. For
example, such an agent may be a peptide which either

2187781

20

comprises, consists of, or consists essentially of a BLM
domain or EGF receptor domains (i.e., domain 1 and
domain 3) or, alternatively, a region of a natural binding
partner or a region of an EGF ligand that binds one or
5   more of these domains, or a fragment of any of these.

In the other preferred embodiments, the methods
or products produce a therapeutic effect. A "therapeutic
effect" generally refers to either the inhibition, to some
extent, of growth of cells causing or contributing to a
10 cell proliferative disorder. A therapeutic effect
relieves to some extent one or more of the symptoms of a
cell proliferative disorder.

In reference to the treatment of a cancer, a
therapeutic effect refers to one or more or the following:
15 1) reduction in tumor size; 2) inhibition (i.e., slowing
to some extent, preferably stopping) of tumor metastasis;
3) inhibition, to some extent, of tumor growth; and/or 4)
relieving to some extent one more or the symptoms
associated with the disorder.
20   In reference to the treatment of a cell prolifer-
ative disorder other than a cancer, a therapeutic effect
refers to either: 1) the inhibition, to some extent, of
the growth of cells causing the disorder; 2) the
inhibition, to some extent, of the production of factors
25 (e.g., growth factors) causing the disorder; and/or
3) relieving to some extent one more or the symptoms
associated with the disorder.

Other features and advantages of the invention
will be apparent from the following description of the
30 preferred embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Fig. 1 is a diagrammatic representation of the
BLM domain shown as regions of identity in the GRB-10,
GRB-7, and F10E9.6 proteins at the amino acid level;

21

Fig. 2 is an alignment of the central domain of GRB-7, GRB-10, F10E9.6 including the BLM domain and within that the pleckstrin domain;

5   Fig. 3 is the amino acid sequence of GRB-7 compared to GRB-10 showing the BLM domain;

Fig. 4 is a diagrammatic representation of a proposed Her-2; GRB-7; and natural binding partner signalling process in breast cancer;

Fig. 5 is a diagrammatic representation of the 10   association between a stationary cell and a cell which is moving.

Figure 6 shows a model for the interaction between EGF and the extracellular domain of EGF receptor. Figure 6A shows that EGF has two binding sites for EGFR 15   and that sEGFR has two binding sites for EGF, as indicated by isothermal titration calorimetry analysis. The high-affinity form of the ligand/receptor complex has a single molecule of EGF bound simultaneously to two receptor molecules. This occurs via the binding of 20   different portions of an EGF molecule (a and b) to different half-sites on each of the two receptor molecules (sites A and B). One of these sites (site A) corresponds to subdomain 3 of the receptor, while site B corresponds to subdomain 1. Thus, the 1:2 EGF:EGFR (high-affinity) 25   complex has a single EGF molecule bound between site A of one receptor and site B of its partner in a dimer (upper ligand molecule in Figure 1). The second (low affinity) event would involve a second EGF molecule interacting with the unoccupied site on each receptor molecule. A slightly 30   unfavorable juxtaposition of the free sites or conformational changes could be responsible for the observed negative cooperativity. The result of the second event would be the arrangement depicted in Figure 1. Additional EGF-EGF interactions, or interactions between 35   the two receptor molecules in the occupied EGFR dimer may also occur in this complex.

2187781

22

Figure 6B shows the similar mode of interaction
between hGH and hGH-receptor. However, while hGH, like
EGF, contains two binding sites for its receptor, the hGH
receptor has only a single binding site for hGH (de Vos et
5   al., *Science* 255:306-312, 1992). Binding of a second hGH
molecule to the complex depicted in Figure 1B must
therefore lead to its dissociation, as is indeed observed
(Cunningham et al., *Science* 254:821-825, 1991; Fuh et al.,
Science 256:1677-1680, 1992). Additional
10 receptor-receptor interactions, not depicted here, were
also observed in the X-ray crystal structure of the 1:2
hGH:hGH-R complex (de Vos et al., *Science* 255:306-312,
1992).

Figure 6C contrasts these situations with one in
15 which the ligand is monovalent, but dimeric, as in PDGF,
CSF-1 or SCF. Independent interaction of the two ligand
protomers with a single binding site in its receptor
results in receptor dimerization and activation by virtue
of the ligand's dimeric nature. Cyt.D. refers to the
20 cytoplasmic domain.

The present invention relates to compositions and
methods for the prevention, prognostic evaluation, and
treatment of oncogenic and other disorders, especially
breast cancer, wherein a protein having a BLM domain or
25 EGF domain is capable of complexing with its natural
binding partner or ligand and is involved in such a
disorder.

Specifically, the present invention relates to
compositions and methods for decreasing or inhibiting the
30 interaction between the components of the two proteins
and/or decreasing or inhibiting the activity of such com-
plexes, and to methods for identifying useful agents for
such compositions. The present invention also relates to
removing or reducing an abnormality in a signal transduc-
35 tion pathway, wherein the signal transduction pathway
contains a protein with a BLM or EGF domain. Further, the
present invention relates to the use of such methods and

23

compositions for the treatment of the oncogenic disorders
of interest, especially breast cancer. The present inven-
tion also relates to compositions and methods for the
treatment of .disorders which involve modulating the
5   activity and/or level of individual components of the
proteins, and relates to methods for the identification of .
agents for such treatments. Additionally, the present
invention relates to methods and compositions for prog-
nostic evaluation of such disorders. Several abbrevia-
10 tions, defined below, are used to describe the present
invention.

<div align="center">ABBREVIATIONS</div>

Amino Acid	One Letter code	Three Letter Code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp
Cysteine	C	Cys
Glutamic Acid	E	Glu
Glutamine	Q	Gln
Glycine	G	Gly
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro

<div align="center">SUBSTITUTE SHEET (RULE 26)</div>

24

Amino Acid	One Letter code	Three Letter Code
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr
Valine	V	Val
Not specified	X	

I.   The BLM Domain

      Referring to Fig. 1, examples of the BLM domain
are shown diagrammatically in three newly characterized

10 proteins. The region shown corresponds to amino acids
between about 200 and 500 in the noted proteins, and
includes the plextrin domain defined by Musacchio et al.,
18 TIBS September 1993 (and references cited therein).
The term "pleckstrin domain or PH domain" is used in this

15 application as defined by Musacchio et al.) Those in the
art will recognize that BLM equivalent domains are readily
identified as those having at least 20% identity in the
amino acid sequence in the noted regions or perform the
same function despite a lower percentage of identity. The

20 percentage of identity between two domains is calculated
by dividing the number of amino acids that are the same in
a given region by the total number of amino acids in the
given region. Proteins including such domains are readily
identified using standard protocols. Peptides containing

25 a BLM domain may be used, for example, in binding
experiments to identify natural binding partners, in the
design of probes for particular proteins, in affinity
columns, or other applications familar to those in the
art. The peptides may also be used in the methods of the

30 invention, for example administration of the peptide may

2187781

25

disrupt or promote the interaction between a BLM domain
and its natural binding partner.

    Other proteins with BLM equivalent domains may be
identified as those having at least 30% similarity or
5 perform the same function despite having an even lower
percentage of similarity. The percentage of similarity is
calculated using a computer program (such as the GCG
Bestfit program) that scores a protein based upon the
number of gaps that must be induced to achieve similarity.

10   Regions that have at least 20% (preferably 30%,
most preferably 60%) identity or 30% (preferably 40%, most
preferably 70%) similarity are recognized as containing
the same domain independent of any knowledge of the
function of the proteins or domains. However, when
15 knowledge regarding the function of the proteins or domain
is known, then equivalent domains may be identified with
much lower identity or similarity (e.g., 5-10% and 10-20%
respectively). For example, the pleckstrin domain
contains two proteins that only share approximately 5%
20 identity to each other. Musacchio, id.

    Those skilled in the art will be able to identify
other proteins with a BLM domain using routine procedures
in the art, such as sequence comparisions, three-
dimensional structure comparisons, analysis of functional
25 activities or properties, etc. For example, many PH
domains have been identified by those in the art in a
short period of time despite the widely divergent
sequences of PH domains and the lack of consensus
regarding the possible function of the PH domain. See,
30 Gibson et al., TIBS, 19:349-353. (1994), incorporated
herein by reference in its entirety, including any
drawings. This analogy is particularly apt, given that
the BLM domain contains a PH domain within it.

    Referring to Fig. 2, the amino acid sequence of
35. the three proteins, shown in Fig. 1, are provided with the
consensus sequence. The standard single letter amino acid
code is used in the figure. The BLM domain is that shown

in GRB-7 between amino acids 95 and 428, and the 5' region
of the BLM domain is that between amino acids 95 and 231.
Thus, the 5' region is 5' of the pleckstrin domain.

Referring to Fig. 3, the protein sequence of GRB-

5 7 and GRB-10 is shown, demonstrating the high degree of
conservation in the BLM domain and pleckstrin domain.

II. BLM Domain Interaction

Referring to Fig. 4, the present invention is
based upon the interaction of the BLM domain of proteins
10 such as GRB-7 with a natural binding partner (shown as a
shaded triangle in the lower portion of the Figure).

Specifically, Fig. 4 shows the interaction of
Her-2 with GRB-7 in the SH2 domain. See, Stein et al., 13
EMBO Journal, 1994 (Appendix A, including figures, hereby
15 incorporated by reference herein). This invention is
designed to measure and/or to affect this interaction
between GRB-7, or an equivalent BLM domain from this or
another protein, and the natural binding partner of that
domain. By such control of the interaction of the BLM
20 domain with its natural binding partner, diseases associ-
ated with problems in cell migration and metastases can be
diagnosed and treated.

For example, referring to Fig. 5 there is shown
a diagrammatic representation of the role of the interac-
25 tion between the BLM domain and its natural binding part-
ner in movement of cells. Specifically, a mobile cell
binds another cell. Without being bound to any particular
theory of the invention, it is presently believed that the
interaction between the BLM domain and its natural ligand
30 may be involved in the signal transduction pathway that
controls the affinity of cell receptors, cell adhesion,
and cell migration.

Isolation of the GRB-7 gene is described in
Margolis et al., (U.S. Patent Application Serial No.
35 07/906,349, filed June 30, 1992) hereby incorporated by
reference. GRB-10 was cloned from λEXlox NIH 3T3 (mouse

2187781

27

fibroblast cell line) using the CORT technique. The probe
was the EGF-Receptor carboxyterminus and the methodology
the same as described for the other GRB's (Skolnik et al.
65 Cell 83-90, 1991; Lowenstein et al., 70 Cell 431, 1992;
5 Margolis et al., PNAS, 89:8894, 1992. The randomly primed
NIH 3T3 library was generated in our laboratory using
standard techniques (Sambrook et al., Molecular Cloning.
A Laboratory Manual, Cold Spring Harbor Laboratory Press,
Cold Spring Harbor, 1989). After the initial clone was
10 isolated, GRB-10 cDNA encoding the full length GRB-10
protein was cloned from the same library using DNA hybrid-
ization as described (Margolis et al., 3 Cell Growth &
Differ., 73, 1992; Margolis et al., 89 Proc. Natl. Acad.
Sci. USA 8894, 1992). The F10E9.6 gene corresponds to
15 that described by Manser and Wood as the mig-10 gene.
Manser and Wood, 11 Developmental Genetics 49, 1990,
hereby incorporated by reference herein.

III.   EGF Receptor Dimerization

    Here we describe studies of the stoichiometry and
20 thermodynamics of binding of the monomeric growth factor
EGF to the soluble extracellular domain of the respective
receptor (which is monomeric). Of the systems in which
ligand:receptor interactions have been studied
extensively, this is among the few cases in which the
25 available data suggest a monovalent mode of interaction
(Weber et al., J. Biol. Chem. 259:14631-14636, 1984).

    We have compared the binding of epidermal growth
factor (EGF) to the soluble extracellular domain of the
respective receptor using isothermal titration calori-
30 metry. EGF shows two modes of binding to its receptor,
one with high affinity (K_D ~ 10 nM) and one with lower
affinity (K_D ~ 200 nM). Surprisingly, each of these
binding modes occurs with a stoichiometry corresponding to
1:2 (EGF:receptor), indicating that the high-affinity
35 complex contains two receptor molecules and a single
ligand, as seen in the case of human growth hormone bound
to its receptor.

28

Binding of EGF to the soluble extracellular
domain caused receptor dimerization as revealed by
treatment with the covalent cross-linking agent DSS and
SDS-PAGE. However, an isolated subdomain (domain 3) of
5 the receptor bound EGF with a simple 1:1 stoichiometry,
and did not dimerize upon binding of EGF. Thus, simulta-
neous binding of a single molecule of EGF to different
regions on two receptor molecules is responsible for
ligand-induced stabilization of the active dimeric form of
10 its receptor.

Isothermal titration calorimetry (ITC) permits
analysis of the stoichiometries of binding. The data
presented here indicate that, while aFGF forms a simple
1:1 complex (K_D = 500 nM) with the extracellular domain of
15 its receptor, EGF initially forms a 1:2 (EGF:sEGFR)
complex (K_D ~ 10 nM). A second molecule of EGF then binds
to this complex with a lower affinity (K_D ~ 200 nM). Thus
EGF binds simultaneously to two receptor molecules, which
may be important for ligand-induced stabilization of the
20 active receptor dimer. In efforts to understand the
mechanism via which binding of a monomeric growth factor
leads to the dimerization of its cognate receptor, we have
compared the stoichiometry and thermodynamic parameters
associated with binding of EGF to soluble forms of the
25 respective receptor.

EGF can induce dimerization of sEGFR in the
absence of accessory molecules (Lax et al., *J. Biol. Chem.*
266:13828-13833, 1991; Hurwitz et al., *J. Biol. Chem.*
266:22035-22043, 1991). This effect is likely to result
30 from ligand-induced conformational changes that stabilize
the receptor dimer and/or multivalent binding of EGF to
its receptor, as has been described for hGH (Cunningham et
al., *Science* 254:821-825, 1991; de Vos et al., *Science*
255:306-312, 1992). The data presented here indicate that
35 multivalent binding is also significant, since a single
molecule of EGF can bind simultaneously to two molecules
of sEGFR. It also appears that sEGFR itself has two

2187781

29

EGF-binding sites, represented by subdomains 1 and 3.
Isolated domain 3 binds EGF in a simple monovalent
fashion.

IV. Models for EGF Binding to sEGFR

5   There are two possible arrangements in which a
single EGF molecule could bind with high-affinity to a
dimer of sEGFR, to form the 1:2 (EGF:sEGFR) complex
reported here. In one scenario, a single EGF binding-site
occurs per receptor molecule, and its occupancy leads to
10   conformational changes in the receptor. These changes, in
turn, lead to association of the occupied receptor with a
second unoccupied receptor molecule. The result is an
asymmetric dimer of the receptor, in which the unoccupied
protomer has a reduced affinity for EGF. This is
15   equivalent to the negative cooperativity observed, for
example, with four successive molecules of NAD⁺ that bind
with   successively   lower   affinity   to   tetrameric
glyceraldehyde-3-phosphate dehydrogenase (Schlessinger and
Levitzki, *J. Mol. Biol.* 82:547-561, 1974). Wofsy et al.
20   (*Biophys. J.* 63:98-110, 1992) have shown that models of
this nature cannot adequately account for the concave-up
curvature, characteristic of negative cooperativity, that
is observed in Scatchard plots obtained in for EGF binding
to its receptor. Our finding that sEGFR itself has two
25   binding sites for EGF thus indicate an alternative model.
       In the alternative scenario, a single EGF mole-
cule would bind simultaneously to two receptor molecules
by interacting with a different site on each. It thus
binds across the dimer interface in a manner similar to
30   that described for hGH binding to hGH-R (de Vos et al.,
Science 255:306-312, 1992), stabilizing the active dimeric
form of the receptor. Titrations of sEGFR into solutions
of EGF showed that two EGF molecules bind to a single
receptor molecule.
35   One site of interaction is subdomain 3 of the
receptor, since it is able to bind EGF in isolation. On

the basis of sequence homology (Lax et al., *Mol. Cell.
Biol.* 8:1970-1978, 1988) and deletion mutagenesis (Lax et
al., *Cell Regulation.* 1:173-188, 1990), we determined that
subdomain 1 represents the other site. Thus, EGF binds to

5   one receptor in the 1:2 (EGF:sEGFR) complex via interac-
tions with subdomains 1 and 3. Interaction with the other
receptor molecule could also occur via subdomain 3.
However, since EGF is an asymmetric molecule, and two EGF
binding sites are present in each sEGFR molecule,

10   interaction with the second receptor is more likely to
occur via subdomain 1, as depicted in Figure 6A. This
suggests one contrast with the hGH/hGH-R system
(Figure 6B).

        While hGH is bivalent, hGH-R is effectively

15   monovalent, since it binds to two different portions of
hGH via essentially the same site on the receptor (de Vos
et al., *Science* 255:306-312, 1992). Formation of a 2:1
(hGH:hGH-R) complex is therefore not possible, and binding
of a second hGH molecule to the 1:2 (hGH:hGH-R) complex

20   must necessarily dissociate the receptor dimer (Cunningham
et al., *Science* 254:821-825, 1991; Fuh et al., *Science*
256:1677-1680, 1992). In the case of EGFR, both ligand
and receptor appear to be bivalent. This being the case,
it is possible that binding of a second EGF molecule to

25   the 1:2 (EGF:sEGFR) complex could occur without
dissociation of the sEGFR dimer, as depicted in Figure 6A.
The second EGF molecule would bind to the unoccupied site
on each receptor molecule.

        The reduced affinity for this second EGF molecule

30   could result from conformational changes in the receptor
that alter the two binding sites. Alternatively, their
position with respect to one another could be restricted
such that simultaneous binding of a single EGF molecule to
both is disfavored. The negative cooperativity that is

35   observed for ATP binding to yeast hexokinase provides an
illustration of this. Each protomer of a hexokinase dimer
contributes two partial sites for ATP binding, and ATP is

bound across the dimer interface via interactions with one
partial site of each protomer (Anderson and Steitz,
J. Mol. Biol. 92:279-287, 1975). The other partial sites
are then positioned such that their simultaneous
5 interaction with ATP is disfavored.

V. Comparison with other Receptors

Recent studies of ligand binding to the aspartate
receptor (Tar) from S. typhimurium (Biemann and Koshland,
Biochemistry 33:629-634, 1994) suggest a model where each
10 receptor dimer has two binding sites for aspartate, each
of which is contributed to by side-chains from both
protomers of the dimer. Two aspartate molecules bind per
dimer, but marked negative cooperativity is observed.
Analysis of the X-ray crystal structure of the Tar
15 periplasmic domain (Milburn et al., *Science* 254:1342-1347,
1991) showed that binding of one aspartate molecule to the
receptor dimer, via interactions with both protomers,
leads to slight conformational changes in the second site.
This is proposed to account for the observed negative
20 cooperativity (Biemann and Koshland, *Biochemistry*
33:629-634, 1994), and is likely to be paralleled in the
case of EGF binding to sEGFR.

A wealth of data also suggest a similar model for
insulin binding to its receptor (de Meyts, "The structural
25 basis of insulin and insulin-like growth factor-I (IGF-I)
receptor binding and negative cooperativity, and its
relevance to mitogenic versus metabolic signaling",
Diabetologia, in press, 1994; Soos et al., *Biochem. J.*
48:419-426, 1993). Both insulin and the insulin receptor
30 α-subunit appear to be bivalent. High-affinity binding of
insulin to its receptor involves the bivalent interaction
of a single insulin molecule with a different site on each
of two α-subunits, spanning the interface of the
pre-existing receptor dimer. Additional insulin molecules
35 can then bind to this complex, although with reduced
affinity as a result of conformational changes induced by

32

binding of the first molecule. (Schaefer et al., *J. Biol. Chem.* 267:23393-23402, 1992).

The model presented here for EGF binding to EGFR suggests novel ways in which receptor antagonists may be
5   designed, which could be of therapeutic value. The rational design of potent antagonists to hGH-R (Fuh et al., *Science* 256:1677-1680, 1992) has been shown to be feasible.

VI.   Thermodynamics of EGF Binding

10   One difference observed between the thermodynamic parameters for EGF binding to sEGFR and the binding of other receptors to their ligands was the different sign of the enthalpy. The interaction is predominantly enthalpy-driven for binding of hGH to its receptor ($\Delta H = -27$
15   kcal/mol) (Cunningham et al., *Science* 254:821-825, 1991), and aspartate binding to Tar of E. coli ($\Delta H = -17$ kcal/mol).

By contrast, EGF binding to sEGFR(1234) had a positive enthalpy value ($\Delta H_1 = 6.8$ kcal/mol $\Delta H_2 = 10.4$
20   kcal/mol). EGF binding to sEGFR(1234) is therefore entropy-driven, implying that the system becomes more disordered upon binding of EGF to sEGFR. Parts of the system that may become more disordered (and negate the negative entropic effect of bringing ligand and macromole-
25   cule together) include the ligand, the macromolecule, and the solvent. One possible explanation for this would be that EGF binding or sEGFR(1234) dimerization results in the burial of significant areas of hydrophobic surface, around which water would be ordered in the unbound state.
30   Release of this water to the bulk would provide an entropic driving force.

That EGF binding to domain 3 has a negative enthalpy ($\Delta H = -2$ kcal/mol) indicates that this does not result directly from EGF binding. The entropic driving
35   force could also arise from a disordering of the receptor structure itself. Indeed, there is evidence for this from

33

studies of the effect of EGF upon the decay of
fluorescence anisotropy suggesting an increase in
flexibility in the receptor upon EGF binding, (Greenfield
et al., *EMBO J.* 8:4115-4123, 1989), whereas a similar
5   analysis of the insulin receptor suggested increased
rigidity upon ligand binding (Schaefer et al., *J. Biol.*
Chem. 267:23393-23402, 1992).

VII.   Compositions

The present invention relates to removing or
10 reducing an abnormality in a signal transduction pathway,
wherein the signal transduction pathway contains an EGF
receptor domain (i.e., domain 1 and/or domain 3) and an
EGF ligand. The present invention also relates to
compositions and methods for the treatment of disorders
15 which involve modulating the activity and/or level of
individual components, and relates to methods for the
identification of agents for such treatments. Addi-
tionally, the present invention relates to methods and
compositions for prognostic evaluation of such disorders.
20   Described herein are compositions and methods for
the prevention, prognostic evaluation, and treatment of
disorders listed herein. Also described are compositions
and methods for the prevention, prognostic evaluation and
treatment of cell proliferative disorders, especially
25 cancer, in which an EGF ligand is involved.
First, methods and compositions for the treatment
of such disorders are described. Such methods and
compositions may include, but are not limited to the
agents capable of decreasing or inhibiting the interaction
30 between EGF receptor domains (i.e., domains 1 and/or 3)
and EGF ligands and agents capable of inhibiting or
decreasing the activity of such complexes, agents capable
of modulating the activity and/or level of individual
components of the proteins, and the use and administration
35 of such agents.

34

Second, methods are described for the identifi-
cation of such agents. These methods may include, for
example, assays to identify agents capable of disrupting
or inhibiting or promoting the interaction between compo-
5   nents of the complexes (e.g., EGF ligand:EGF receptor
complexes), and may also include paradigms and strategies
for the rational design of drugs capable of disruption
and/or inhibition and/or promotion of such complexes.

VIII. Ligand/Receptor Complexes
10   The complexes involved in the invention include
proteins with an EGF ligand domain or proteins with an EGF
receptor domain or derivatives thereof, as described below
and also include at least one member of the BLM domain-
containing family of proteins, and/or a derivative
15   thereof, and at least one member of a natural binding
ligand containing protein or a derivative thereof, as
described below. Under standard physiological conditions,
the components of such complexes are capable of forming
stable, non-covalent attachments with one or more of the
20   other complex components. Methods for the purification
and production of such protein complexes, and of cells
that exhibit such complexes are described below.

IX. Disruption of Protein Complexes
         Disruption of BLM domain-containing associated
25   protein complexes, for example by decreasing or inhibiting
the interactions between component members such a complex
may have differing modulatory effects on the event
involved, depending on the individual protein complex.
         "Disruption", as used here, is meant to refer not
30   only to a physical separation of protein complex compo-
nents, but also refers to a perturbation of the activity
of the complexes, regardless of whether or not such com-
plexes remain able, physically, to form.
         "Activity", as used here, refers to the function
35   of the protein complex in the signal transduction cascade

35

of the cell in which such a complex is formed, i.e.,
refers to the function of the complex in effecting or
inhibiting a transduction of an extracellular signal into
a cell. For example, the effect of complex disruption may
5   augment, reduce, or block a signal normally transduced
into the cell. Likewise, depending on the disorder
involved, either augmentation, reduction, or blockage of
a signal normally transduced into the cell will be desir-
able for the treatment of the disorder.

10   A disorder involving a complex may, for example,
develop because the presence of such a complex brings
about the aberrant inhibition of a normal signal transduc-
tion event. In such a case, the disruption of the complex
would allow the restoration of the usual signal transduc-
15   tion event. Further, an aberrant complex may bring about
an altered subcellular adaptor protein localization, which
may result in, for example, dysfunctional cellular events.
An inhibition of the complex in this case would allow for
restoration or maintenance of a normal cellular architec-
20   ture. Still further, an agent or agents that cause(s)
disruption of the complex may bring about the disruption
of the interactions among other potential components of a
complex.

       Nucleotide sequences encoding peptide agents
25   which are to be utilized intracellularly may be expressed
in the cells of interest, using techniques which are well
known to those of ordinary skill in the art. For example,
expression vectors derived from viruses such as retro-
viruses, vaccinia virus, adenoviruses, adeno-associated
30   virus, herpes viruses, or bovine papilloma virus, may be
used for delivery and expression of such nucleotide
sequences into the targeted cell population. Methods for
the construction of such vectors are well known. See, for
example, the techniques described in Maniatis et al.,
35   1989, Molecular Cloning A Laboratory Manual, Cold Spring
Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current
Protocols in Molecular Biology, Greene Publishing Associ-

36

ates and Wiley Interscience; N.Y. Complex-binding domains
can be identified using, for example, techniques such as
those described in Rotin et al. (Rotin, D. et al., EMBO J.
11:559-567), Songyang et al. (Songyang, S. et al., 1993,
5 Cell 72:767-778); Felder, S. et al., 1993, Mol. Cell.
Biol. 13:1449-1455, Fantl, W.J. et al., 1992, Cell 69:413-
422, and Domchek, S.M. et al., 1992, Biochemistry 31:9865-
9870.

Alternatively, antibodies capable of interfering
10 with complex formation may be produced as described below
and administered for the treatment of disorders involving
a component capable of forming a complex with another
protein. For example, neutralizing antibodies which are
capable of interfering with ligand binding may be admin-
15 istered using standard techniques. Alternatively, nucleo-
tide sequences encoding single-chain antibodies may be
expressed within the target cell population by utilizing,
for example, techniques such as those described in Marasco
et al. (Marasco, W. et al., 1993, Proc. Natl. Acad. Sci.
20 USA 90:7889-7893).

Agents which act intracellularly to interfere
with the formation and/or activity of the protein com-
plexes of the invention may also be small organic or
inorganic compounds. A method for identifying these and
25 other intracellular agents is described below.

X. Antibodies to Complexes

Described herein are methods for the production
of antibodies which are capable of specifically recogniz-
ing a complex or an epitope thereof, or of specifically
30 recognizing an epitope on either of the components of the
complex, especially those epitopes which would not be
recognized by the antibody when the component is present
separate and apart from the complex. Such antibodies may
include, but are not limited to polyclonal antibodies,
35 monoclonal antibodies (mAbs), humanized or chimeric anti-
bodies, single chain antibodies, Fab fragments, F(ab')$_2$

37

fragments, fragments produced by a FAb expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a complex in a

5 biological sample, or, alternatively, as a method for the inhibition of a complex formation, thus, inhibiting the development of a disorder.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of

10 animals immunized with an antigen, such as a complex, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, various host animals may be immunized by injection with the complex including but not limited to rabbits, mice, rats, etc. Various

15 adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin,

20 dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and <u>Corynebacterium</u> <u>parvum</u>.

A monoclonal antibody, which is a substantially homogeneous population of antibodies to a particular

25 antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (Nature 256:495-497 (1975) and U.S. Patent No. 4,376,110), the

30 human B-cell hybridoma technique (Kosbor et al., 1983, Immunology Today 4:72; Cole et al., 1983 Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies

35 may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated

38 .

in vitro or _in vivo_. Production of high titers of mAbs
in vivo makes this the presently preferred method of
production.

5   In addition, techniques developed for the produc-
tion of "chimeric antibodies" (Morrison et al., 1984,
Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al.,
1984, Nature, 312:604-608; Takeda et al., 1985, Nature,
314:452-454) by splicing the genes from a mouse antibody
molecule of appropriate antigen specificity together with

10   genes from a human antibody molecule of appropriate biolo-
gical activity can be used. A chimeric antibody is a
molecule in which different portions are derived from dif-
ferent animal species, such as those having a variable
region derived from a murine mAb and a human immunoglobu-

15   lin constant region.

       Alternatively, techniques described for the
production of single chain antibodies (U.S. Patent
4,946,778; Bird, 1988, Science 242:423-426; Huston et al.,
1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward

20   et al., 1989, Nature 334:544-546) can be adapted to
produce complex-specific single chain antibodies. Single
chain antibodies are formed by linking the heavy and light
chain fragment of the Fv region via an amino acid bridge,
resulting in a single chain polypeptide.

25   Antibody fragments which contain specific binding
sites of a complex may be generated by known techniques.
For example, such fragments include but are not limited
to: the F(ab')$_2$ fragments which can be produced by pepsin
digestion of the antibody molecule and the Fab fragments

30   which can be generated by reducing the disulfide bridges
of the F(ab')$_2$ fragments. Alternatively, Fab expression
libraries may be constructed (Huse et al., 1989, Science,
246:1275-1281) to allow rapid and easy identification of
monoclonal Fab fragments with the desired specificity to

35   the PTK/adaptor complex.

       One or more components of a protein complex may
be present at a higher than normal cellular level (_i.e._,

39

higher than the concentration known to usually be present
in the cell type exhibiting the protein complex of inter-
est) and/or may exhibit an abnormally increased level of
cellular activity (i.e., greater than the activity known
5 to usually be present in the cell type exhibiting the
protein complex of interest).

For example, the gene encoding a protein complex
component may begin to be overexpressed, or may be ampli-
fied (i.e., its gene copy number may be increased) in
10 certain cells, leading to an increased number of component
molecules within these cells. Additionally, a gene encod-
ing a protein complex component may begin to express a
modified protein product that exhibits a greater than
normal level of activity. "Activity", here, refers to the
15 normal cellular function of the component, either enzy-
matic or structural whose function may include, for
example, bringing two or more cellular molecules into the
appropriate proximity.

Such an increase in the cellular level and/or
20 activity of a protein complex may lead to the development
of a disorder. Treatment of such disorders may, there-
fore, be effectuated by the administration of agents which
decrease the cellular level and/or the activity of the
overexpressed and/or overactive protein complex component.
25 Techniques for decreasing the cellular level and/or the
activity of one or more of the protein complex components
of interest may include, but are not limited to antisense
or ribozyme approaches, and/or gene therapy approaches,
each of which is well known to those of skill in the art.
30 XI. Antisense and Ribozyme Approaches

Included in the scope of the invention are oligo-
ribonucleotides, including antisense RNA and DNA molecules
and ribozymes that function to inhibit translation of one
or more components of a protein complex. Anti-sense RNA
35 and DNA molecules act to directly block the translation of
mRNA by binding to targeted mRNA and preventing protein
translation. With respect to antisense DNA, oligodeoxy-

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ribonucleotides derived from the translation initiation
site, e.g., between -10 and +10 regions of the relevant
nucleotide sequence, are preferred.

Ribozymes are enzymatic RNA molecules capable of
5 catalyzing the specific cleavage of RNA. The mechanism of
ribozyme action involves sequence specific interaction of
the ribozyme molecule to complementary target RNA, fol-
lowed by a endonucleolytic cleavage. Within the scope of
the invention are engineered hammerhead or other motif
10 ribozyme molecules that specifically and efficiently
catalyze endonucleolytic cleavage of RNA sequences encod-
ing protein complex components.

Specific ribozyme cleavage sites within any
potential RNA target are initially identified by scanning
15 the target molecule for ribozyme cleavage sites which
include the following sequences, GUA, GUU and GUC. Once
identified, short RNA sequences of between 15 and 20
ribonucleotides corresponding to the region of the target
gene containing the cleavage site may be evaluated for
20 predicted structural features, such as secondary struc-
ture, that may render the oligonucleotide sequence unsuit-
able. The suitability of candidate targets may also be
evaluated by testing their accessibility to hybridization
with complementary oligonucleotides, using ribonuclease
25 protection assays. See, Draper PCT WO 93/23569.

Both anti-sense RNA and DNA molecules and ribo-
zymes of the invention may be prepared by any method known
in the art for the synthesis of RNA molecules. See,
Draper, id. hereby incorporated by reference herein.
30 These include techniques for chemically synthesizing
oligodeoxyribonucleotides well known in the art such as
for example solid phase phosphoramidite chemical synthe-
sis. Alternatively, RNA molecules may be generated by
in vitro and in vivo transcription of DNA sequences encod-
35 ing the antisense RNA molecule. Such DNA sequences may be
incorporated into a wide variety of vectors which incorpo-
rate suitable RNA polymerase promoters such as the T7 or

41

SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

5   Various modifications to the DNA molecules may be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribo- or deoxy- nucleotides to the 5' and/or 3' ends of

10   the molecule or the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages within the oligo-deoxyribonucleotide backbone.

XII. Gene Therapy

        Target cell populations may be modified by intro-

15   ducing altered forms of one or more components of the protein complexes in order to modulate the activity of such complexes. For example, by reducing or inhibiting a complex component activity within target cells, an abnormal signal transduction event(s) leading to a condi-

20   tion may be decreased, inhibited, or reversed. Deletion or missense mutants of a component, that retain the ability to interact with other components of the protein complexes but cannot function in signal transduction may be used to inhibit an abnormal, deleterious signal

25   transduction event.

        Expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of nucleotide sequences encoding recom-

30   binant protein complex components into the targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences. See, for example, the techniques described in Maniatis et al., 1989, Mole-

35   cular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current Protocols in Molecular Biology, Greene Publishing Associ-

ates and Wiley Interscience, N.Y. Alternatively, recombi-
nant nucleic acid molecules encoding protein sequences can
be used as naked DNA or in reconstituted system e.g.,
liposomes or other lipid systems for delivery to target
5   cells (See e.g., Felgner et. al., 1989, Nature 337:387-8).
XIII.   Pharmaceutical Formulations and Modes of
          Administration

          The particular compound, antibody, antisense or
ribozyme molecule that affects the protein complexes and
10 the disorder of interest can be administered to a patient
either by themselves, or in pharmaceutical compositions
where it is mixed with suitable carriers or excipient(s).
In treating a patient exhibiting a disorder of interest,
a therapeutically effective amount of a agent or agents
15 such as these is administered.   A therapeutically
effective dose refers to that amount of the compound that
results in amelioration of symptoms or a prolongation of
survival in a patient.

          Toxicity and therapeutic efficacy of such com-
20 pounds can be determined by standard pharmaceutical proce-
dures in cell cultures or experimental animals, e.g., for
determining the LD_{50} (the dose lethal to 50% of the popula-
tion) and the ED_{50} (the dose therapeutically effective in
50% of the population). The dose ratio between toxic and
25 therapeutic effects is the therapeutic index and it can be
expressed as the ratio LD_{50}/ED_{50}. Compounds which exhibit
large therapeutic indices are preferred.   The data
obtained from these cell culture assays and animal studies
can be used in formulating a range of dosage for use in
30 human.   The dosage of such compounds lies preferably
within a range of circulating concentrations that include
the ED_{50} with little or no toxicity. The dosage may vary
within this range depending upon the dosage form employed
and the route of administration utilized. ..

35   For any compound used in the method of the inven-
tion, the therapeutically effective dose can be estimated
initially from cell culture assays. For example, a dose

43

can be formulated in animal models to achieve a circulat-
ing plasma concentration range that includes the IC_{50} as
determined in cell culture (i.e., the concentration of the
test compound which achieves a half-maximal disruption of
5 the protein complex, or a half-maximal inhibition of the
cellular level and/or activity of a complex component).
Such information can be used to more accurately determine
useful doses in humans. Levels in plasma may be measured,
for example, by HPLC. The exact formulation, route of
10 administration and dosage can be chosen by the individual
physician in view of the patient's condition. (See e.g.
Fingl et al., 1975, in "The Pharmacological Basis of
Therapeutics", Ch. 1 p1).

      It should be noted that the attending physician
15 would know how to and when to terminate, interrupt, or
adjust administration due to toxicity, or to organ dys-
functions. Conversely, the attending physician would
also know to adjust treatment to higher levels if the
clinical response were not adequate (precluding toxicity).
20 The magnitude of an administered dose in the management
of the oncogenic disorder of interest will vary with the
severity of the condition to be treated and to the route
of administration. The severity of the condition may, for
example, be evaluated, in part, by standard prognostic
25 evaluation methods. Further, the dose and perhaps dose
frequency, will also vary according to the age, body
weight, and response of the individual patient. A program
comparable to that discussed above may be used in veteri-
nary medicine.

30   Depending on the specific conditions being
treated, such agents may be formulated and administered
systemically or locally. Techniques for formulation and
administration may be found in "Remington's Pharmaceutical
Sciences," 1990, 18th ed., Mack Publishing Co., Easton,
35 PA. Suitable routes may include oral, rectal, trans-
dermal, vaginal, transmucosal, or intestinal administra-
tion; parenteral delivery, including intramuscular, subcu-

taneous, intramedullary injections, as well as intra-
thecal, direct intraventricular, intravenous, intraperi-
toneal, intranasal, or intraocular injections, just to
name a few. For injection, the agents of the invention

5　may be formulated in aqueous solutions, preferably in
physiologically compatible buffers such as Hanks's
solution, Ringer's solution, or physiological saline
buffer. For such transmucosal administration, penetrants
appropriate to the barrier to be permeated are used in the

10　formulation. Such penetrants are generally known in the
art.

Use of pharmaceutically acceptable carriers to
formulate the compounds herein disclosed for the practice
of the invention into dosages suitable for systemic admin-

15　istration is within the scope of the invention. With
proper choice of carrier and suitable manufacturing prac-
tice, the compositions of the present invention, in parti-
cular, those formulated as solutions, may be administered
parenterally, such as by intravenous injection. The

20　compounds can be formulated readily using pharmaceutically
acceptable carriers well known in the art into dosages
suitable for oral administration. Such carriers enable
the compounds of the invention to be formulated as tab-
lets, pills, capsules, liquids, gels, syrups, slurries,

25　suspensions and the like, for oral ingestion by a patient
to be treated.

Agents intended to be administered intracellu-
larly may be administered using techniques well known to
those of ordinary skill in the art. For example, such

30　agents may be encapsulated into liposomes, then admin-
istered as described above. Liposomes are spherical lipid
bilayers with aqueous interiors. All molecules present in
an aqueous solution at the time of liposome formation are
incorporated into the aqueous interior. The liposomal

35　contents are both protected from the external microen-
vironment and, because liposomes fuse with cell membranes,
are efficiently delivered into the cell cytoplasm. Addi-

45

tionally, due to their hydrophobicity, small organic
molecules may be directly administered intracellularly.

Pharmaceutical compositions suitable for use in
the present invention include compositions wherein the
5   active ingredients are contained in an effective amount to
achieve its intended purpose. Determination of the effec-
tive amounts is well within the capability of those
skilled in the art, especially in light of the detailed
disclosure provided herein. In addition to the active
10 ingredients, these pharmaceutical compositions may contain
suitable pharmaceutically acceptable carriers comprising
excipients and auxiliaries which facilitate processing of
the active compounds into preparations which can be used
pharmaceutically. The preparations formulated for oral
15 administration may be in the form of tablets, dragees,
capsules, or solutions.

The pharmaceutical compositions of the present
invention may be manufactured in a manner that is itself
known, e.g., by means of conventional mixing, dissolving,
20 granulating, dragee-making, levigating, emulsifying, encap-
sulating, entrapping or lyophilizing processes.

Pharmaceutical formulations for parenteral admin-
istration include aqueous solutions of the active com-
pounds in water-soluble form. Additionally, suspensions
25 of the active compounds may be prepared as appropriate
oily injection suspensions. Suitable lipophilic solvents
or vehicles include fatty oils such as sesame oil, or
synthetic fatty acid esters, such as ethyl oleate or
triglycerides, or liposomes. Aqueous injection suspen-
30 sions may contain substances which increase the viscosity
of the suspension, such as sodium carboxymethyl cellulose,
sorbitol, or dextran. Optionally, the suspension may also
contain suitable stabilizers or agents which increase the
solubility of the compounds to allow for the preparation
35 of highly concentrated solutions.

Pharmaceutical preparations for oral use can be
obtained by combining the active compounds with solid

46 .

excipient, optionally grinding a resulting mixture, and
processing the mixture of granules, after adding suitable
auxiliaries, if desired, to obtain tablets or dragee
cores. Suitable excipients are, in particular, fillers
5   such as sugars, including lactose, sucrose, mannitol, or
sorbitol; cellulose preparations such as, for example,
maize starch, wheat starch, rice starch, potato starch,
gelatin, gum tragacanth, methyl cellulose, hydroxypropyl-
methyl-cellulose, sodium carboxymethylcellulose, and/or
10   polyvinylpyrrolidone (PVP). If desired, disintegrating
agents may be added, such as the cross-linked polyvinyl
pyrrolidone, agar, or alginic acid or a salt thereof such
as sodium alginate.

      Dragee cores are provided with suitable coatings.
15   For this purpose, concentrated sugar solutions may be
used, which may optionally contain gum arabic, talc, poly-
vinyl pyrrolidone, carbopol gel, polyethylene glycol,
and/or titanium dioxide, lacquer solutions, and suitable
organic solvents or solvent mixtures. Dyestuffs or pig-
20   ments may be added to the tablets or dragee coatings for
identification or to characterize different combinations
of active compound doses.

      Pharmaceutical preparations which can be used
orally include push-fit capsules made of gelatin, as well
25   as soft, sealed capsules made of gelatin and a plasti-
cizer, such as glycerol or sorbitol. The push-fit
capsules can contain the active ingredients in admixture
with filler such as lactose, binders such as starches,
and/or lubricants such as talc or magnesium stearate and,
30   optionally, stabilizers. In soft capsules, the active
compounds may be dissolved or suspended in suitable
liquids, such as fatty oils, liquid paraffin, or liquid
polyethylene glycols. In addition, stabilizers may be
added.

35   **XIV. Identification of Agents**

      The complexes, components of such complexes,
functional equivalents thereof, and/or cell lines that

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47

express such components and exhibit such protein complexes
may be used to screen for additional compounds, anti-
bodies, or other molecules capable of modulating the
signal transduction event such complexes are involved in.
5 Methods for purifying and/or producing such complexes,
components of the complexes, functional equivalents
thereof, and/or cell lines are described herein. The
compounds, antibodies, or other molecules identified may,
for example, act to disrupt the protein complexes of the
10 invention (i.e., decrease or inhibit interactions between
component members of the complexes, thereby causing phy-
sical separation of the components, and/or perturbing the
activity of the complexes) or may lower the cellular level
and/or decrease the activity of one or more of the compo-
15 nents of such complexes.

Such compounds may include, but are not limited
to, peptides made of D- and/or L-configuration amino acids
(in, for example, the form of random peptide libraries;
see Lam, K.S. et al., 1991, Nature 354:82-84), phosphopep-
20 tides (in, for example, the form of random or partially
degenerate, directed phosphopeptide libraries, see
Songyang, Z. et al., 1993, Cell 767-778), antibodies, and
small organic or inorganic molecules. Synthetic com-
pounds, natural products, and other sources of potentially
25 biologically active materials may be screened in a variety
of ways, as described herein. The compounds, antibodies,
or other molecules identified may be used as oncogenic
disorder treatments, as described herein.

Compounds that bind to individual components, or
30 functional portions of the individual components of the
complexes (and may additionally be capable of disrupting
complex formation) may be identified.

One such method included within the scope of the
invention is a method for identifying an agent to be
35 tested for an ability to modulate a signal transduction
pathway disorder comprising:

(a) exposing at least one agent to a protein
comprising a functional portion of a member of the roteine
complex for a time sufficient to allow binding of the
agent to the functional portion of the member;

5   (b) removing non-bound agents; and

(c) determining the presence of the compound
bound to the functional portion of the member of the
protein complex, thereby identifying an agent to be tested
for an ability to modulate a disorder involving a
10 polypeptide complex.

By "signal transduction disorder" is meant any
disease or condition associated with an abnormality in a
signal transduction pathway. The protein complex referred
to below is a physical association of a protein containing
15 a BLM domain and its natural binding partner. The level
of interaction between the two components of the complex
may be abnormal and thus cause the abnormality in the
signal transduction pathway. Alternatively, the level of
interaction between the complex components may be normal,
20 but affecting that interaction may effectively treat a
signal transduction pathway disorder.

The terms "protein", "polypeptide" and "peptide"
refer to a compound formed of 5-50 or more amino acids
joined together by peptide bonds. These terms do not
25 carry precise length connotations and as used herein the
terms may refer to any amino acid sequence of five or more
residues. No implication regarding the relative length of
a sequence is intended by the use one term rather than the
other. An "amino acid" is a subunit that is polymerized
30 to form proteins and there are twenty amino acids that are
universally found in proteins. The general formula for an
amino acid is $H_2N-CHR-COOH$, in which the R group can be
anything from a hydrogen atom (as in the amino acid
glycine) to a complex ring (as in the amino acid
35 tryptophan).

A functional portion of an individual component
of the complexes may be defined here as a protein portion

of an individual component of a complex still capable of
forming a stable complex with another member of the com-
plex under standard cellular and physiological conditions.
For example, a functional portion of a component may
5   include, but is not limited to, a protein portion of the
BLM domain which is still capable of stably binding a
corresponding natural binding domain of an associated
protein, and thus is still capable of forming a complex
with that protein. Further, in the case of the catalytic
10 domains of the individual components of the invention, a
functional portion of a catalytic domain may refer to a
protein still capable of stably binding a substrate mole-
cule under standard physiological conditions.

One method utilizing this approach that may be
15 pursued in the isolation of such complex component-binding
molecules would include the attachment of a component mole-
cule, or a functional portion thereof, to a solid matrix,
such as agarose or plastic beads, microtiter wells, petri
dishes, or membranes composed of, for example, nylon or
20 nitrocellulose, and the subsequent incubation of the
attached component molecule in the presence of a potential
component-binding compound or compounds. Attachment to
said solid support may be direct or by means of a compo-
nent specific antibody bound directly to the solid
25 support. After incubation, unbound compounds are washed
away, component-bound compounds are recovered. By utiliz-
ing this procedure, large numbers of types of molecules
may be simultaneously screened for complex component-
binding activity.

30   The complex components which may be utilized in
the above screening method may include, but are not lim-
ited to, molecules or functional portions thereof, such as
catalytic domains, phosphorylation domains, extracellular
domains, or portions of extracellular domains, such as
35 ligand-binding domains, and adaptor proteins, or func-
tional portions thereof. The peptides used may be phos-
phorylated, e.g., may contain at least one phosphorylated

amino acid residue, preferably a phosphorylated Tyr amino
acid residue, or may be unphosphorylated. A phosphoryla-
tion domain may be defined as a peptide region that is
specifically phosphorylated at certain amino acid resi-
5   dues. A functional portion of such a phosphorylation
domain may be defined as a peptide capable of being speci-
fically phosphorylated at certain amino acids by a
specific protein.

        Molecules exhibiting binding activity may be
.10 further screened for an ability to disrupt protein com-
plexes. Alternatively, molecules may be directly screened
for an ability to promote the complexes. For example,
in vitro complex formation may be assayed by, first,
immobilizing one component, or a functional portion
15   thereof, of the complex of interest to a solid support.
Second, the immobilized complex component may be exposed
to a compound such as one identified as above, and to the
second component, or a functional portion thereof, of the
complex of interest. Third, it may be determined whether
20   or not the second component is still capable of forming a
complex with the immobilized component in the presence of
the compound. In addition, one could look for an increase
in binding.

        Additionally, complex formation in a whole cell
25   may be assayed by utilizing co-immunoprecipitation tech-
niques well known to those of skill in the art. Briefly,
a cell line capable of forming a complex of interest may
be exposed to a compound such as one identified as above,
and a cell lysate may be prepared from this exposed cell
30   line. An antibody raised against one of the components of
the complex of interest may be added to the cell lysate,
and subjected to standard immunoprecipitation techniques.
In cases where a complex is still formed, the
immunoprecipitation will precipitate the complex, whereas
35   in cases where the complex has been disrupted, only the
complex component to which the antibody is raised will be
precipitated.

51

A preferred method for assessing modulation of
complex formation within a cell utilizes a method similar
to that described above. Briefly, a cell line capable of
forming a complex of interest is exposed to a test com-
5   pound. The cells are lysed and the lysate contacted with
an antibody specific to one component of the complex, said
antibody having been previously bound to a solid support.
Unbound material is washed away, and the bound material is
exposed to a second antibody, said second antibody binding
10   specifically to a second component of the complex. The
amount of second antibody bound is easily detected by
techniques well known in the art. Cells exposed to an
inhibitory test compound will have formed a lesser amount
of complex compared to cells not exposed to the test
15   compound, as measured by the amount of second antibody
bound. Cells exposed to a test compound that promotes
complex formation will have an increased amount of second
antibody bound.

The effect of an agent on the transformation capa-
20   bility of the complex of interest may be directly assayed.
Such agents may, but are not required to, include those
agents identified by utilizing the above screening tech-
nique. For example, an agent or agents may be admini-
stered to a cell such as a breast cancer cell, capable of
25   forming a complex, for example, which, in the absence of
any inhibitory agent, would lead to the cell's transfor-
mation. The transformation state of the cell may then be
measured _in vitro_, by monitoring, for example, its ability
to form colonies in soft agar. Alternatively, a cell's
30   transformation state may be monitored _in vivo_ by, for
example, determining its ability to form tumors in
immunodeficient nude or severe combined immunodeficiency
(SCID) mice.

Agents capable of disrupting complex formation
35   and capable of reducing or inhibiting disorders, such as
breast cancer; which involve the formation of such com-
plexes may be used in the treatment of patients exhibiting

or at risk for such disorders. A sufficient amount of
agent or agents such as those described above may be
administered to a patient so that the oncogenic capability
of cells which, in the absence of such agents, would

5   contain protein complexes, is reduced or eliminated.
    XV. Purification and Production of Complexes

        Described in this Section are methods for the
    synthesis or recombinant expression of components, or
    fragments thereof, of the protein complexes of the inven-

10 tion. Also described herein are methods by which cells
    exhibiting the protein complexes of the invention may be
    engineered.
    XVI. Purification Methods

        The complexes of the invention may be substan-

15 tially purified, i.e., may be purified away from at least
    90% (on a weight basis), and from at least 99%, if
    desired, of other proteins, glycoproteins, and other
    macromolecules with which it is associated. Such purifi-
    cation can be achieved by utilizing a variety of proce-

20 dures well known to those of skill in the art, such as
    subjecting cells, tissue or fluid containing the complex
    to a combination of standard methods, for example, ammo-
    nium sulfate precipitation, molecular sieve chromato-
    graphy, and/or ion exchange chromatography.

25   Alternatively, or additionally, a complex may be
    purified by immunoaffinity chromatography using an immuno-
    adsorbent column to which an antibody is immobilized which
    is capable of binding to one or more components of the
    complex. Such an antibody may be monoclonal or poly-

30 clonal in origin. Other useful types of affinity purifi-
    cation for the protein complex may utilize, for example,
    a solid-phase substrate which binds the catalytic kinase
    domain of a protein, or an immobilized binding site for
    noncatalytic domains of the components of the complex,

35 which bind in such a manner as to not disrupt the complex.
        The complex of the present invention may be
    biochemically purified from a variety of cell or tissue

sources. For purification of a naturally occurring com-
plex, cellular sources may include, for example, baculo-
virus-infected SF9 cells, A-431, CHO, and/or 3T3 cells.
In a preferred embodiment of the present invention, the

5   complex comprises the receptor GRB-7 or GRB-10 adaptor
protein.

XVII.   Synthesis and Expression Methods

Methods for the synthesis of polypeptides or
fragments thereof, which are capable of acting as com-

10   ponents of the complexes of the present invention, are
well-known to those of ordinary skill in the art. See,
for example, Creighton, 1983, Proteins: Structures and
Molecular Principles, W.H. Freeman and Co., NY, which is
incorporated herein, by reference, in its entirety.

15   - -   Components of a complex which have been sepa-
rately synthesized or recombinantly produced, may be
reconstituted to form a complex by standard biochemical
techniques well known to those skilled in the art. For
example, samples containing the components of the complex

20   may be combined in a solution buffered with greater than
about 150mM NaCl, at a physiological pH in the range of 7,
at room temperature. For example, a buffer comprising
20mM Tris-HCl, pH 7.4, 137mMNaCl, 10% glycerol, 1% Triton
X-100, 0.1% SDS, 0.5% deoxycholate and 2mM EDTA could be

25   used.

Methods for preparing the components of complexes
of the invention by expressing nucleic acid encoding pro-
teins are described herein. Methods which are well known
to those skilled in the art can be used to construct

30   expression vectors containing protein coding sequences and
appropriate transcriptional/translational control signals.
These methods include, for example, _in vitro_ recombinant
DNA techniques, synthetic techniques and _in vivo_ recombi-
nation/genetic recombination. DNA and RNA synthesis may,

35   additionally, be performed using an automated synthe-
sizers. See, for example, the techniques described in
Maniatis et al., 1989, Molecular Cloning A Laboratory

2187781

54

Manual, Cold Spring Harbor Laboratory, N.Y. and Ausubel
et al., 1989, Current Protocols in Molecular Biology,
Greene Publishing Associates and Wiley Interscience, N.Y.

      A variety of host-expression vector systems may
5   be utilized to express the coding sequences of the compo-
nents of the complexes of the invention. Such host-
expression systems represent vehicles by which the coding
sequences of interest may be produced, but also represent
cells which may, when transformed or transfected with the
10   appropriate nucleotide coding sequences, exhibit the
protein complexes of the invention. These include but are
not limited to microorganisms such as bacteria (e.g.,
E.coli, B. subtilis) transformed with recombinant bacteri-
ophage DNA, plasmid DNA or cosmid DNA expression vectors
15   containing protein coding sequences; yeast (e.g.,
Saccharomyces and Pichia) transformed with recombinant
yeast expression vectors containing the protein coding
sequences; insect cell systems infected with recombinant
virus expression vectors (e.g., baculovirus) containing
20   the protein coding sequences; plant cell systems infected
with recombinant virus expression vectors (e.g., cauli-
flower mosaic virus, CaMV; tobacco mosaic virus, TMV) or
transformed with recombinant plasmid expression vectors
(e.g., Ti plasmid) containing the protein coding sequences
25   coding sequence; or mammalian cell systems (e.g., COS,
CHO, BHK, 293, 3T3) harboring recombinant expression con-
structs containing promoters derived from the genome of
mammalian cells (e.g., metallothionein promoter) or from
mammalian viruses (e.g., the adenovirus late promoter; the
30   vaccinia virus 7.5K promoter).

      In bacterial systems a number of expression
vectors may be advantageously selected depending upon the
use intended for the complex being expressed. For
example, when large quantities of complex proteins are to
35   be produced for the generation of antibodies or to screen
peptide libraries, vectors which direct the expression of
high levels of fusion protein products that are readily

purified may be desirable. Such vectors include but are
not limited to the E. coli expression vector pUR278
(Ruther et al., 1983, EMBO J. 2:1791), in which the
protein coding sequence may be ligated individually into
5 the vector in frame with the lac Z coding region so that
a fusion protein is produced; pIN vectors (Inouye &
Inouye, 1985, Nucleic acids Res. 13:3101-3109; Van Heeke
& Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the
like. pGEX vectors may also be used to express foreign
10 polypeptides   as   fusion   proteins   with   glutathione
S-transferase (GST). In general, such fusion proteins are
soluble and can easily be purified from lysed cells by
adsorption to glutathione-agarose beads followed by
elution in the presence of free glutathione. The pGEX
15 vectors are designed to include thrombin or factor Xa
protease cleavage sites so that the cloned protein can be
released from the GST moiety.

In an insect system, Autographa californica
nuclear polyhedrosis virus (AcNPV) is used as a vector to
20 express foreign genes. The virus grows in Spodoptera
frugiperda cells. The complex coding sequence may be
cloned   individually   into   non-essential   regions   (for
example the polyhedrin gene) of the virus and placed under
control of an AcNPV promoter (for example the polyhedrin
25 promoter). Successful insertion of the PTK/adaptor com-
plex coding sequence will result in inactivation of the
polyhedrin gene and production of non-occluded recombinant
virus (i.e., virus lacking the proteinaceous coat coded
for by the polyhedrin gene). These recombinant viruses
30 are then used to infect Spodoptera frugiperda cells in
which the inserted gene is expressed (e.g., see Smith
et al., 1983, J. Viol. 46:584; Smith, U.S. Patent No.
4,215,051).

In mammalian host cells, a number of viral based
35 expression systems may be utilized. In cases where an
adenovirus is used as an expression vector, the complex
coding sequence may be ligated to an adenovirus transcrip-

2187781

56

tion/translation control complex, e.g., the late promoter
and tripartite leader sequence. This chimeric gene may
then be inserted in the adenovirus genome by _in vitro_ or
in vivo recombination. Insertion in a non-essential
5   region of the viral genome (e.g., region E1 or E3) will
result in a recombinant virus that is viable and capable
of expressing proteins in infected hosts. (E.g., See
Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-
3659). Specific initiation signals may also be required
10 for efficient translation of inserted coding sequences.
These signals include the ATG initiation codon and
adjacent sequences.

     In cases where an entire protein gene, including
its own initiation codon and adjacent sequences, is
15 inserted into the appropriate expression vector, no
additional translational control signals may be needed.
However, in cases where only a portion of the coding
sequence is inserted, exogenous translational control
signals, including the ATG initiation codon, must be
20 provided. Furthermore, the initiation codon must be in
phase with the reading frame of the desired coding
sequence to ensure translation of the entire insert.
These exogenous translational control signals and initia-
tion codons can be of a variety of origins, both natural
25 and synthetic. The efficiency of expression may be
enhanced by the inclusion of appropriate transcription
enhancer elements, transcription terminators, etc. (see
Bittner et al., 1987, Methods in Enzymol. 153:516-544).

     In addition, a host cell strain may be chosen
30 which modulates the expression of the inserted sequences,
or modifies and processes the gene product in the specific
fashion desired. Such modifications (e.g., glycosylation)
and processing (e.g., cleavage) of protein products may be
important for the function of the protein. Different host
35 cells have characteristic and specific mechanisms for the
post-translational processing and modification of
proteins. Appropriate cells lines or host systems can be

chosen to ensure the correct modification and processing
of the foreign protein expressed. To this end, eukaryotic
host cells which possess the cellular machinery for proper
processing of the primary transcript, glycosylation, and
5 phosphorylation of the gene product may be used. Such
mammalian host cells include but are not limited to CHO,
VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombi-
nant proteins, stable expression is preferred. For
10 example, cell lines which stably coexpress both the
proteins may be engineered. Rather than using expression
vectors which contain viral origins of replication, host
cells can be transformed with the protein encoding DNA
independently or coordinately controlled by appropriate
15 expression control elements (e.g., promoter, enhancer,
sequences, transcription terminators, polyadenylation
sites, etc.), and a selectable marker.

Following the introduction of foreign DNA, engi-
neered cells may be allowed to grow for 1-2 days in an
20 enriched media, and then are switched to a selective
media. The selectable marker in the recombinant plasmid
confers resistance to the selection and allows cells to
stably integrate the plasmid into their chromosomes and
grow to form foci which in turn can be cloned and expanded
25 into cell lines. This method may advantageously be used
to engineer cell lines which coexpress both the PTK and
adaptor protein. Such engineered cell lines are particu-
larly useful in screening and evaluation of compounds that
affect signals mediated by the complexes.
30 A number of selection systems may be used,
including but not limited to the herpes simplex virus
thymidine kinase (Wigler, et al., 1977, Cell 11:223),
hypoxanthine-guanine phosphoribosyltransferase (Szybalska
& Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026),
35 and adenine phosphoribosyltransferase (Lowy, et al., 1980,
Cell 22:817) genes can be employed in tk⁻, hgprt⁻ or aprt⁻
cells, respectively. Also, antimetabolite resistance can

58

be used as the basis of selection for dhfr, which confers
resistance to methotrexate (Wigler, et al., 1980, Natl.
Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl.
Acad. Sci. USA 78:1527); gpt, which confers resistance to
5 mycophenolic acid (Mulligan & Berg, 1981), Proc. Natl.
Acad. Sci. USA 78:2072); neo, which confers resistance to
the aminoglycoside G-418 (Colberre-Garapin, et al., 1981,
J. Mol. Biol. 150:1); and hygro, which confers resistance
to hygromycin (Santerre, et al.; 1984, Gene 30:147) genes.
10   New members of the protein families capable of
forming the complexes of the invention may be identified
and isolated by molecular biological techniques well known
in the art. For example, a previously unknown protein
encoding gene may be isolated by performing a polymerase
15 chain reaction (PCR) using two degenerate oligonucleotide
primer pools designed on the basis of highly conserved
sequences within domains common to members of the protein
family.
     The template for the reaction may be cDNA
20 obtained by reverse transcription of mRNA prepared from
cell lines or tissue known to express complexes. The PCR
product may be subcloned and sequenced to insure that the
amplified sequences represent the sequences of a member of
the PTK or adaptor subfamily. The PCR fragment may then
25 be used to isolate a full length protein cDNA clone by
radioactively labeling the amplified fragment and screen-
ing a bacteriophage cDNA library. Alternatively, the
labeled fragment may be used to screen a genomic library.
For a review of cloning strategies which may be used, see
30 e.g., Maniatis, 1989, Molecular Cloning, A Laboratory Man-
ual, Cold Springs Harbor Press, N.Y.; and Ausubel et al.,
1989, Current Protocols in Molecular Biology, (Green
Publishing Associates and Wiley Interscience, N.Y.).
     A general method for cloning previously unknown
35 proteins has been described by Skolnik (Skolnik, E.Y.,
1991, Cell 65:75) and Skolnik et al., (U.S. Patent Appli-
cation Serial No. 07/643,237) which are incorporated

2187781

herein, by reference, in their entirety, including draw-
ings. Briefly, new members of the family of proteins may
be identified by their ability to specifically bind to at
least a portion of a BLM or EGF domain in a peptide.

5   XVIII. Derivatives of Complexes

Also provided herein are functional derivatives
of a complex. By "functional derivative" is meant a
"chemical derivative," "fragment," "variant," "chimera,"
or "hybrid" of the complex, which terms are defined below.

10 A functional derivative retains at least a portion of the
function of the protein, for example reactivity with an
antibody specific for the complex, enzymatic activity or
binding activity mediated through noncatalytic domains,
which permits its utility in accordance with the present

15 invention.

A "chemical derivative" of the complex contains
additional chemical moieties not normally a part of the
protein. Covalent modifications of the protein complex or
peptides are included within the scope of this invention.

20 Such modifications may be introduced into the molecule by
reacting targeted amino acid residues of the peptide with
an organic derivatizing agent that is capable of reacting
with selected side chains or terminal residues, as
described below.

25   Cysteinyl residues most commonly are reacted with
alpha-haloacetates (and corresponding amines), such as
chloroacetic acid or chloroacetamide, to give carboxy-
methyl or carboxyamidomethyl derivatives. Cysteinyl resi-
dues also are derivatized by reaction with bromotrifluoro-

30 acetone, α-bromo-β(5-imidozoyl)propionic acid, chloro-
acetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl
disulfide, methyl 2-pyridyl disulfide, p-chloromercuri-
benzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-
nitrobenzo-2-oxa-1,3-diazole.

35   Histidyl residues are derivatized by reaction
with diethylprocarbonate at pH 5.5-7.0 because this agent
is relatively specific for the histidyl side chain. Para-

2187781

60

bromophenacyl bromide also is useful; the reaction is
preferably performed in 0.1 M sodium cacodylate at pH 6.0.

     Lysinyl and amino terminal residues are reacted
with succinic or other carboxylic acid anhydrides. Deri-
5 vatization with these agents has the effect or reversing
the charge of the lysinyl residues. Other suitable
reagents for derivatizing α-amino-containing residues
include imidoesters such as methyl picolinimidate; pyri-
doxal phosphate; pyridoxal; chloroborohydride; trinitro-
10 benzenesulfonic acid; O-methylisourea; 2,4 pentanedione;
and transaminase-catalyzed reaction with glyoxylate.

     Arginyl residues are modified by reaction with
one or several conventional reagents, among them phenyl-
glyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and nin-
15 hydrin. Derivatization of arginine residues requires that
the reaction be performed in alkaline conditions because
of the high pK_a of the guanidine functional group. Further-
more, these reagents may react with the groups of lysine
as well as the arginine ε-amino group.

20   Tyrosyl residues are well-known targets of modi-
fication for introduction of spectral labels by reaction
with aromatic diazonium compounds or tetranitromethane.
Most commonly, N-acetylimidizol and tetranitromethane are
used to form O-acetyl tyrosyl species and 3-nitro
25 derivatives, respectively.

     Carboxyl side groups (aspartyl or glutamyl) are
selectively modified by reaction carbodiimide (R'-N-C-N-
R') such as 1-cyclohexyl-3-(2-morpholinyl(4-ethyl) carbo-
diimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbo-
30 diimide. Furthermore, aspartyl and glutamyl residue are
converted to asparaginyl and glutaminyl residues by reac-
tion with ammonium ions.

     Glutaminyl and asparaginyl residues are fre-
quently deamidated to the corresponding glutamyl and
35 aspartyl residues. Alternatively, these residues are
deamidated under mildly acidic conditions. Either form of
these residues falls within the scope of this invention.

61

Derivatization with bifunctional agents is useful, for example, for cross-linking the component peptides of the complexes to each other or the complex to a water-insoluble support matrix or to other macromole-
5   cular carriers.   Commonly used cross-linking agents include, for example, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic .acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-
10   dithiobis(succinimidylpropionate),   and   bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[p-azidophenyl) dithiolpropioimidate yield photoactivatable intermediates that. are capable of forming crosslinks in the presence of light.
15   Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.
20   Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains (Creighton, T.E., Proteins: Structure and Molecular Proper-
25   ties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the Nterminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.
       Such derivatized moieties may improve the stability, solubility, absorption, biological half life, and
30   the like.   The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein complex and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 18th ed., Mack Publishing Co., Easton,
35   PA (1990).
       The term "fragment" is used to indicate a poly-peptide derived from the amino acid sequence of the pro-

teins, of the complexes having a length less than the
full-length polypeptide from which it has been derived.
Such a fragment may, for example, be produced by proteo-
lytic cleavage of the full-length protein. Preferably,
5 the fragment is obtained recombinantly by appropriately
modifying the DNA sequence encoding the proteins to delete
one or more amino acids at one or more sites of the
C-terminus, N-terminus, and/or within the native sequence.

     Fragments of a protein, when present in a complex
10 resembling the naturally occurring complex, are useful for
screening for compounds that act to modulate signal trans-
duction, as described below. It is understood that such
fragments, when present in a complex may retain one or
more characterizing portions of the native complex.
15 Examples of such retained characteristics include: cata-
lytic activity; substrate specificity; interaction with
other molecules in the intact cell; regulatory functions;
or binding with an antibody specific for the native
complex, or an epitope thereof.
20   Another functional derivative intended to be
within the scope of the present invention is a complex
comprising at least one "variant" polypeptide which either
lack one or more amino acids or contain additional or
substituted amino acids relative to the native polypep-
25 tide. The variant may be derived from a naturally occur-
ring complex component by appropriately modifying the
protein DNA coding sequence to add, remove, and/or to
modify codons for one or more amino acids at one or more
sites of the C-terminus, N-terminus, and/or within the
30 native sequence. It is understood that such variants
having added, substituted and/or additional amino acids
retain one or more characterizing portions of the native
complex, as described above.

     A functional derivative of complexes comprising
35 proteins with deleted, inserted and/or substituted amino
acid residues may be prepared using standard techniques
well-known to those of ordinary skill in the art. For

example, the modified components of the functional deriva-
tives may be produced using site-directed mutagenesis
techniques (as exemplified by Adelman et al., 1983, DNA
2:183) wherein nucleotides in the DNA coding the sequence
5 are modified such that a modified coding sequence is modi-
fied, and thereafter expressing this recombinant DNA in a
prokaryotic or eukaryotic host cell, using techniques such
as those described above. Alternatively, components of
functional derivatives of complexes with amino acid dele-
10 tions, insertions and/or substitutions may be conveni-
ently prepared by direct chemical synthesis, using methods
well-known in the art. The functional derivatives of the
complexes typically exhibit the same qualitative biologi-
cal activity as the native complexes.

15 <u>XIX. Evaluation of Disorders</u>

        The protein complexes of the invention involved
in disorders may be utilized in developing a prognostic
evaluation of the condition of a patient suspected of
exhibiting such a disorder.   For example, biological
20 samples obtained from patients suspected of exhibiting a
disorder involving a protein complex may be assayed for
the presence of such complexes. If such a protein complex
is normally present, and the development of the disorder
is caused by an abnormal quantity of the complex, the
25 assay should compare complex levels in the biological
sample to the range expected in normal tissue of the same
cell type.

        Among the assays which may be undertaken may
include, but are not limited to isolation of the protein
30 complex of interest from the biological sample, or assay-
ing for the presence of the complex by exposing the sample
to an antibody specific for the complex, but non-reactive
to any single, non-complexed component, and detecting
whether antibody has specifically bound.

35   Alternatively, one or more of the components of
the protein complex may be present in an abnormal level or
in a modified form, relative to the level or form expected

64 -

is normal, nononcogenic tissue of the same cell type. It
is possible that overexpression of both components may
indicate a particularly aggressive disorder. Thus, an
assessment of the individual and levels of mRNA and pro-
5   tein in diseased tissue cells may provide valuable clues
as to the course of action to be undertaken in treatment
of such a disorder. Assays of this type are well known to
those of skill in the art, and may include, but are not
limited to, Northern blot analysis, RNAse protection
10 assays, and PCR for determining mRNA levels. Assays
determining protein levels are also well known to those of
skill in the art, and may include, but are not limited to,
Western blot analysis, immunoprecipitation, and ELISA
analysis. Each of these techniques may also reveal poten-
15 tial differences in the form (e.g., the primary, second-
ary, or tertiary amino acid sequence, and/or post-transla-
tional modifications of the sequence) of the component(s).

Disorders

HER2 driven disorders are characterized by over-
20 activity of HER2. Over-activity of HER2 refers to either
an amplification of the gene encoding HER2 or the
production of a level of HER2 activity which can be
correlated with a cell proliferative disorder (i.e., as
the level of HER2 increases the severity of one or more of
25 the symptoms of the cell proliferative disorder
increases).

Activation of HER2 activity can result from
several different events including: 1) ligand binding; 2)
HER2 stimulation through transphosphorylation by activated
30 EGFR; and 3) overexpression of her-2, encoding HER2
protein, can create a high abundance of HER2 resulting in
ligand-independent dimerization. HER2 activity can be
assayed by measuring one or more of the following
activities: (1) phosphorylation of HER2; (2)
35 phosphorylation of a HER2 substrate (e.g., IP, kinase, and
PI 4-kinase, see Scott et al., Journal of Biological
22:14300, 1991); (3) activation of an HER2 adapter

molecule; and (4) increased cell division.   These
activities can be measured using techniques described   -
below and known in the art.   For example, IP_3 kinase, and
PI 4-kinase, activities can be assayed as described by
5   Scott *et al.*, *supra*, cell division can be assayed by
measuring ^3H-thymidine incorporation into DNA, and
phosphorylation of HER2 can be assayed as described by
Gazit *et al.*, *J. Med. Chem.* *34*:1896-1907, 1991, or as
described in examples detailed below.

10   Treatment of patients suffering from a HER2
disorder is facilitated by first determining whether the
cell proliferative disorder is characterized by an over-
activity of HER2.   After the disorder is identified,
patients suffering from such a disorder can be identified
15 by analysis of their symptoms by procedures well known to
medical doctors.   Such identified patients can then be
treated as described herein.

           HER2 driven disorders are typically cell
proliferative disorders such as cancers.   HER2 driven
20 disorders appear to be responsible for a sub-population of
different types of cancers.   For example, as noted above,
Slamon *et al.*, found about 30% of breast cancer cells to
have increased HER2 gene expression.   Slamon *et al.*, also
found a correlation between *her2* (*c-erbB-2*) amplification
25 and poor patient prognosis.

           The use of the present invention to treat breast
cancer is preferred because of the prevalence and severity
of breast cancer.   Carcinoma of the breast is the most
common cancer among women and their second leading cause
30 of cancer death (Marshall, E., *Science*   *259*:618-621,
1993).   The incidence of breast cancer has been increasing
over the past several decades (Marshall, *supra*, and
Harris, J.R., et al, *New Engl. J . Med.*, *327(5)*:319-328,
1992).

35   In addition to breast cancers, increased HER2
activity or gene expression has been associated with
certain types of stomach adenocarcinomas, salivary gland

66 .

adenocarcinomas, endometrial cancers, ovarian adenocarcinomas, gastric cancers, colorectal cancers, non-small cell lung cancer, and glioblastomas. The methods described herein can be used to identify the sub-
5   populations of these different cancers which are characterized by over-activity of HER2. A cancer cell refers to various types of malignant neoplasms, most of which can invade surrounding tissues, and may metastasize to different sites, as defined by Stedman's Medical
10   Dictionary 25th edition (Hensyl ed. 1990).

Some of the featured compounds can be used to treat cell proliferative disorders characterized by inappropriate EGFR activity. "Inappropriate EGFR" activity refers to either 1) EGF-receptor (EGFR)
15   expression in cells which normally do not express EGFR; 2) EGF expression by cells which normally do not express EGF; 3) increased EGF-receptor (EGFR) expression leading to unwanted cell proliferation; 4) increased EGF expression leading to unwanted cell proliferation; and/or 5) muta-
20   tions leading to constitutive activation of EGF-receptor (EGFR). The existence of inappropriate or abnormal EGF and EGFR levels or activities is determined by procedures well known in the art.

An increase in EGF activity or expression is
25   characterized by an increase in one or more of the activities which can occur upon EGF ligand binding such as: (1) auto-phosphorylation of EGFR, (2) phosphorylation of an EGFR substrate (e.g., PLCγ, see Fry supra) , (3) activation of an adapter molecule, and/or (4) increased
30   cell division. These activities can be measured using techniques described below and known in the art. For example auto-phosphorylation of EGFR can be measured as described in the examples below using an anti-phospho-tyrosine antibody, and increased cell division can be
35   performed by measuring ^{3}H-thymidine incorporation into DNA. Preferably, the increase in EGFR activity is characterized

67

by an increased amount of phosphorylated EGFR and/or DNA synthesis.

Unwanted cell proliferation can result from inappropriate EGFR activity occurring in different types
5 of cells including cancer cells, cells surrounding a cancer cell, and endothelial cells. Examples of disorders characterized by inappropriate EGF activity include cancers such as glioma, head, neck, gastric, lung, breast, ovarian, colon, and prostate; and other types of cell
10 proliferative disorders such as psoriasis.

Psoriasis is a common, chronic disease of the skin consisting of erythematous papules which coalesce to form plaques with distinct borders. As the disease progresses and if it is untreated, a silvery, yellow-white
15 scale develops. New lesions tend to appear at sites of trauma; they may be in any location, but frequently are located on the scalp, knees, elbows, umbilicus, and genitalia. The clinical course is variable but less than one-half of the patients followed for a prolonged period
20 will have prolonged remissions. Severity may range from a minimal cosmetic problem to a life-threatening emergency. In about 5% of cases arthritis will develop and, in most of these, joint involvement will occur after the onset of the skin lesions. The course of the
25 arthritis is mild, affects only a few joints, and spontaneous remissions occur. Most of the skin changes, with the exception of inflammation, may be explained by the rapid turnover of the epidermis. Normal skin produces about 1250 cells a day for each square cm, and these come
30 from 27,000 cells; psoriatic skin produces 35,000 new cells each day for each square cm, and these come from 52,000 cells. The normal duration of the cell cycle of skin is 311 hours, but is reduced to 36 hours for psoriatic skin.

68

EXAMPLES

The examples below are non-limiting and are
merely representative of various aspects and features of
the present invention. The examples below demonstrate

5 the mechanism of EGF receptor dimerization, using vari-
ous methods, including isothermal titration calorimetry.

We describe here studies of the stoichiometry
of growth factor binding to this receptor, and compare
its characteristics using isothermal titration

10 calorimetry (ITC). This technique gives information not
only concerning the binding constant of the interaction,
but also provides a direct measurement of the enthalpy
(ΔH) and stoichiometry of binding. We have generated
milligram quantities of the extracellular ligand-binding

15 domains of the EGF receptor in CHO cells or insect cells
for biophysical studies (Lax et al., J. Biol. Chem.
266:13828-13833, 1991; Hurwitz et al., J. Biol. Chem.
266:22035-22043, 1991.
Production of soluble forms of the EGF-receptor

20 extracellular domain

The various forms of sEGFR were expressed in CHO
cells which were transfected with a CVN expression
vector containing the cDNA for the desired fragment of
EGFR. Generation of the construct encoding sEGFR was

25 previously described (Lax et al., J. Biol. Chem.
266:13828-13833, 1991). The construct encoding
sEGFR(123) was generated by introducing a stop codon
into this construct at the position corresponding to
residue 506 of EGFR.

30   Purification of sEGFR and sEGFR(123) was
performed by immunoaffinity chromatography using the
mAb108 antibody, as described (Hurwitz et al., J. Biol.
Chem. 266:22035-22043, 1991). After affinity purifi-
cation, sEGFR was concentrated and loaded onto a

35 Superose 6 (Pharmacia) gel-filtration column. The
purity of the resulting preparations was estimated by
Coomassie Blue-staining of overloaded SDS-PAGE gels to

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69

be better than 95%. sEGFR contains residues 1-624 of
the mature receptor, while sEGFR(123) contains residues
1-505.

       Domain 3 of sEGFR was prepared by limited
5 proteolysis of sEGFR with proteinase K, with subsequent
purification of the fragment as previously described
(Kohda et al., *J. Biol. Chem.* 268:1976-1981, 1993). The
resulting approximately 35 kDa (glycosylated) fragment
includes residues 302-505 of EGFR, including the com-
10 plete subdomain 3 as originally defined by Lax et al.
(*Mol. Cell. Biol.* 8:1831-1834, 1988), which encompasses
residues 310-474.

       hEGF was purchased from Intergen, and used
without further purification. Analytical gel-filtration
15 and SDS-PAGE suggested that this is also better than 95%
pure. Mouse EGF (K21) was a gift from Dr. E. Nice,
Ludwig Institute, Melbourne, Australia.

       Determination of the concentration of EGF and
the various forms of sEGFR was achieved by measurement
20 of OD at 278 nM. For initial titrations, the extinction
coefficient was estimated from the number of tryptophan
and tyrosine residues in the respective proteins. Quan-
titative amino-acid analysis of several samples with
known OD_{278} was then performed to determine the correct
25 extinction coefficient. ε_{278} for hEGF was 14400 $M^{-1} cm^{-1}$,
ε_{278} for mouse EGF (K21) was 6000 $M^{-1} cm^{-1}$, ε_{278} for sEGFR
was 58500 $M^{-1} cm^{-1}$, ε_{278} for sEGFR(123) was 46000 $M^{-1} cm^{-1}$,
and ε_{278} for sEGFR (3) was 16400 $M^{-1} cm^{-1}$.

Chemical cross-linking experiments

30   Studies of the ability of EGF (20 μM) to
enhance covalent cross-linking of the various forms of
sEGFR (10 μM) were performed using the cross-linking
reagent disuccinimidyl suberate (DSS), as previously
described (Lax et al., *J. Biol. Chem.* 266:13828-13833,
35 1991b). The reaction products were analyzed by SDS-PAGE
and stained with Coomassie Blue.

Binding studies employing isothermal titration
calorimetry (ITC)

      All experiments were performed using the OMEGA
instrument from MicroCal Inc., Northampton, MA, as

5 described in detail by Wiseman et al. (Anal. Biochem.
179:131-137, 1989). In a typical titration, EGF was
added over sixteen injections (of volume 15 µl) to the
portion of the EGF-receptor extracellular domain under
study, which was present in the isothermal calorimeter

10 cell at 25 °C at the concentration given in Tables 1 and
2. In every titration, the concentrations of reactants
were sufficient to result in saturation of all potential
binding sites, as evidenced by the tendency of the
heat-change per injection to zero.

15   Heats of dilution for both reactants (growth-
factor and receptor) with buffer solution were measured
by titration of buffer into a solution of the receptor
extracellular domain, as well as of growth factor into
protein-free buffer solution. The total measured heats

20 were corrected for these heats of dilution prior to data
analysis. Given the unusual nature of the titrations
observed in these studies, we paid particular attention
to the determination of the heats of dilution of the
reactants. The heats per injection were equal for any

25 given dilution experiment.

      We also established that the time interval
between injections was sufficient to allow equilibrium
to be reached for all the reported titrations. For each
titration, both reactants were dialyzed exhaustively (in

30 the same vessel) against the buffer used for the titra-
tion (10 mM HEPES, pH 7.4, 100 mM NaCl, 3.4 mM EDTA).

      Titration curves were fit using the ORIGIN
software (MicroCal Inc., Northampton, MA.), to give the
parameters quoted in Table 1 fits to the titration data,

35 yielding reliable estimates of K_B, can be obtained for
c-values between about 1.0 and approximately 200
(Wiseman et al., Anal. Biochem. 179:131-137, 1989).

This presented a problem for ITC analysis of the EGF/sEGFR interaction, since two events occur which differ in their K_a values by about one order of magnitude, and the associated enthalpies are relatively
5   small.

     C-values in the case of EGF/sEGFR(1234) titrations are in the range of 15 - 52 for the second binding event (K_{a2}), which is ideal, but are in the region of 300 - 1000 for the first (high-affinity) event
10   (K_{a1}). This is reflected in the large errors associated with the K_{a1} values in Table 2, whereas the errors associated with K_{a2} are reasonable. Experiments performed under conditions where c for the high-affinity event was less than 100 gave titrations that could not be fit
15   adequately. Therefore, values for the high-affinity binding constants reported here should be regarded as approximate K_a values. It should be noted that these considerations do not affect the ability to fit for ΔH and stoichiometry values.

20   Example 1:   Two Different Modes for EGF Binding to sEGFR

     To compare the characteristics of EGF binding to its receptor with those observed for aFGF binding to sFGFR, recombinant human EGF was titrated into a solu-
25   tion of sEGFR(1234), which includes the entire extracellular domain of the receptor (residues 1-624; with all four proposed subdomains present; Lax et al., *Cell Regulation* 2:337-345, 1991; Hurwitz et al., *J. Biol. Chem.* 266:22035-22043, 1991; Lax et al., *EMBO J.* 8:421-427,
30   1989). These titrations showed unexpected characteristics.

     The overall stoichiometry of EGF binding was determined to be 1:1, in agreement with previous studies (Weber et al., *J. Biol. Chem.* 259:14631-14636, 1984;
35   Günther et al., *J. Biol. Chem.* 265:22082-22085, 1990). However, the shape of the titration curves clearly shows

72

that more than one binding event occurs during the
titration. The titration curves obtained for EGF
binding to sEGFR could not be fit satisfactorily by
assuming a single class of binding site. The best fits
5   to the data in this case were obtained by assuming that
two types of binding site exist on the receptor, each
with a different affinity for EGF. There is a great
deal of evidence for the existence of two different
classes of EGF-binding sites presented by the
10   EGF-receptor on the cell-surface.

            This heterogeneity gives rise to the curved
Scatchard plots that have been observed in studies of
EGF to cells or isolated EGFR (Bôni-Schnetzler and
Pilch, *Proc. Natl. Acad. Sci. USA* 84:7832-7836, 1987;
15   Yarden and Schlessinger, *Biochemistry* 26:1434-1442,
1987; Wofsy et al., *Biophys. J.* 63:98-110, 1992). In
the context of the receptor-dimerization-model for
transmembrane signaling, it is thought that the high-
affinity form of the receptor represents its active,
20   dimeric form (Yarden and Schlessinger, *Biochemistry*
26:1434-1442, 1987; Yarden and Schlessinger,
Biochemistry 26:1443-1451, 1987. A number of recent
studies provide strong support for this suggestion (Zhou
et al., *Biochemistry* 32:8193-8198, 1993; Sorokin et al.,
25   *J. Biol. Chem.* 269:9752-9759, 1994).

            The nature of the two different modes of bind-
ing of EGF to sEGFR can be understood by reference to
Table 2. The best fits to the binding data suggest that
a high-affinity ($K_m \sim 10$ nM) binding event, with a
30   moderately positive value for ΔH, (site 1) is followed
in the titration by a second, lower affinity ($K_m \sim 200$
nM), event with a higher positive value for ΔH (site 2).
The binding isotherms thus appear to be the result of
two equilibrium binding events.

35   Titration data was obtained for EGF binding to
sEGFR, and for the binding of EGF to sEGFR. In these
titrations, the best fits were obtained using a model

based upon two independent classes of binding site. The thermodynamic parameters in each case are given in Tables 2 and 3. Titration of 16 x 15 μl aliquots of recombinant human EGF (85 μM) into sEGFR (13 μM) at 25°C

5 was performed and titration of 16 x 15 μl aliquots of recombinant truncated (K21) mouse EGF (394 μM) into sEGFR (28 μM) at 25°C was also performed. In addition, titration of 20 x 10 μl aliquots of sEGFR (160 μM) into human EGF (17 μM) at 25°C was performed.

10   Upon injection of the first aliquots of EGF into the sEGFR solution, the high-affinity reaction will predominate (binding to site 1). This has the smaller (positive) associated enthalpy value (+7 kcal/mol). During subsequent injections these high-affinity sites

15 begin to saturate, and the second equilibrium reaction begins (binding to site 2). EGF that is injected into the calorimeter cell at this stage will partition between the two sites, since their affinities differ by only an order of magnitude. As the titration pro-

20 gresses, an increasing proportion of the injected EGF will bind to the lower affinity site (site 2), which has a larger (positive) enthalpy value (+10 kcal/mol). Therefore, the heat absorbed per injection will increase accordingly.

25   The heat per injection will reach its maximum value when the first site is completely saturated, and all EGF that is injected binds to site 2 (which has the highest positive enthalpy). Thereafter, the heat absorbed per injection will fall as site 2 begins to

30 becomes saturated. Finally, the heat absorbed tends to zero when no further detectable ligand binding occurs. Titrations exhibiting this behavior were obtained in more than three separate experiments using hEGF. The same behavior was also observed in two experiments in

35 which the binding of a truncated (residues 1-49) recom-binant mouse EGF from a different source was studied.

Example 2:   Stoichiometry of EGF Binding to sEGFR

74

The occurrence of two different modes of EGF
binding to sEGFR is entirely consistent with previous
data regarding the occurrence of high- and low-affinity
forms of EGFR. The ratio of the affinities of the two
5   events seen in these studies is similar to that observed
for EGFR on the cell surface and in detergent solution.
Moreover, the K_D values estimated here are similar in
magnitude to those previously reported in studies of EGF
binding to sEGFR (Lax et al., *J. Biol. Chem.* 266:13828-
10   13833, 1991b; Hurwitz et al., *J. Biol. Chem.* 266:22035-
22043, 1991; Greenfield et al., *EMBO J.* 8:4115-4123,
1989) as well as to the full-length receptor solubilized
in detergent (Yarden et al., *J. Biol. Chem.* 260:315-319,
1985; Yarden and Schlessinger, *Biochemistry* 26:1434-
15   1442, 1987).

The surprising observation, however, in the
present studies is that each mode of binding occurs with
a stoichiometry of 1:2 (EGF:sEGFR), resulting in a final
complex with overall stoichiometry of 1:1 (Table 1).
20   Thus there appears to be one high-affinity and one low-
affinity binding-site per receptor dimer. Indeed, in
the titrations performed in Example 1, the inflection
point at which the first equilibrium reaction is com-
plete (where the heat per injection is maximal) occurs
25   at an EGF:sEGFR ratio of approximately 0.6. The high-
affinity EGF-binding site therefore appears to be
composed of two sEGFR molecules. A similar situation
has been described for binding of the hGH to its
receptor (Cunningham et al., *Science* 254:821-825, 1991),
30   also employing ITC, and the simultaneous binding of two
receptor molecules to a single ligand molecule has been
confirmed in this case by X-ray crystallographic studies
(de Vos et al., *Science* 255:306-312, 1992).

After saturation of the high-affinity 1:2
35   EGF:sEGFR complex suggested by these experiments, a
second EGF molecule binds to give an overall 1:1 stoi-
chiometry, in agreement with that measured previously

under conditions of excess EGF (Weber et al., *J. Biol.*
Chem. 259:14631-14636, 1984; Günther et al., *J. Biol.*
Chem. 265:22082-22085, 1990). It is not possible to
determine whether this second binding event leads to the
5   formation of a 2:2 EGF:sEGFR complex, or to dissociation
of the complex into two binary EGF:sEGFR complexes. It
has been reported that binary (1:1) complexes of hGH
with hGH-R result when hGH is added to its receptor in
large excess (Cunningham et al., *Science* 254:821-825,
10   1991).

Example 3:   A Single sEGFR can Bind Two EGF Molecules

     Titrations of sEGFR into solutions of EGF
resulted in binding curves with similar characteristics.
The best fits to the data thus suggest that two binding
15   events also occur in these titrations. The first event
occurs with a stoichiometry of 2:1 (EGF:sEGFR),
suggesting that two EGF molecules can bind
simultaneously to a single sEGFR molecule. The second,
lower affinity, event corresponds to the formation of an
20   EGF/sEGFR complex in which the overall stoichiometry is
1:1, as was also observed as the final stage when EGF
was titrated into a solution of sEGFR. A third event,
which would correspond to the formation of a 1:2
EGF:sEGFR complex, is not observed in titrations of
25   sEGFR into EGF. Titrations of EGF into sEGFR also
showed no evidence for a third event that would corres-
pond to formation of the 2:1 (EGF:sEGFR) complex.

     Similar observations have been reported by
Cunningham et al. (*Science* 254:821-825, 1991) for hGH
30   binding to the soluble portion of hGH-R. A fluorescence
quenching study showed that addition of hGH in large
excess to the 1:2 (hGH:hGH-R) complex resulted in its
dissociation into two binary 1:1 complexes. This event
was not detectable in the ITC experiments reported in
35   this study (Cunningham et al., 1991), suggesting either
that the enthalpy per injection associated with the
event is below the limit of detection of the calori-

meter, or that the excess of hGH required for its
occurrence was not reached.

We suggest that similar possibilities explain
our failure to detect the expected third event in ITC
5 studies of the EGF/sEGFR interaction. In titrations of
EGF into sEGFR, the ratio of the concentration of EGF to
that of sEGFR in the calorimeter cell ranged from around
0.1 to 2.5. Similarly, in titrations of sEGFR into EGF,
the sEGFR:EGF concentration ratio ranged from 0.1 to
10 1.8. Increasing the maximum ratio in either set of
titrations, in order to observe the proposed third
event, would not yield interpretable data. Finally,
analysis of an EGF:sEGFR complex formed with an excess
of EGF, and isolated by gel filtration, also suggested a
15 state in which two EGF molecules were bound to each
sEGFR.

Example 4:   **Binding of EGF to Different Portions of**
              sEGFR

The calorimetric studies of the EGF/sEGFR
20 interaction presented above indicate the existence of
complexes in which a single EGF molecule can bind to two
receptor molecules. They also indicate a state in which
two EGF molecules can bind to a single receptor mole-
cule. One possible explanation for these observations
25 would be that each receptor has two different potential
EGF-binding domains, or half-sites. In the 2:1
EGF:sEGFR complex, each would be occupied by a separate
ligand. In the 1:2 EGF:sEGFR complex, a different half-
site on each receptor molecule could interact with the
30 same EGF molecule.

The extracellular domain of EGFR can be divided
into four subdomains: 1, 2, 3, and 4, on the basis of
internal sequence homology (Lax et al., *EMBO J.* 8:421-
427, 1989). Domains 2 and 4 are the cysteine-rich
35 domains, and domains 1 and 3 are defined above. Studies
of ligand binding to chimeric chicken/human EGF-receptor
molecules, exploiting the differences in specificity of

the two different receptors, indicate that domain 3 is a
major determinant of ligand-binding domain in the
receptor ((Lax et al., *EMBO J.* 8:421-427, 1989)), and
that domain 1 also makes some contribution (Lax et al.,

5 *Cell Regulation* 2:337-345, 1991a). The role of domain 3
has been confirmed by affinity labeling studies (Lax et
al., *Mol. Cell. Biol.* 8:1831-1834, 1988b), and by the
observation that EGF binds specifically to isolated
domain 3 (Kohda et al., *J. Biol. Chem.* 268:1976-1981,

10 1993). Significant homology is observed between
subdomains 1 and 3 (Lax et al., *Mol. Cell. Biol.*
8:1970-1978, 1988), and deletion of domain 1 has been
reported to reduce the affinity of EGF for the receptor
by approximately one order of magnitude (Lax et al.,

15 *Cell Regulation* 1:173-188, 1990).

In order to investigate the possibility that
subdomains 1 and 3 of sEGFR correspond to the potential
half-sites for EGF binding, we generated two additional
fragments of sEGFR: sEGFR(123), which lacks the second

20 cysteine-rich domain (domain 4), and includes residues
1-505; as well as sEGFR(3), which contains only
subdomain 3 (for example, residues 302-505). These
definitions of the subdomains are merely meant to
clarify what sequences were used in the examples and is

25 not meant to limit the claims in any manner. sEGFR(3)
has previously been shown to bind EGF; but no
ligand-induced dimerization was observed (Kohda et al.,
J. Biol. Chem. 268:1976-1981, 1993).

EGF induced dimerization of sEGFR(123) but not

30 sEGFR(3). Purified sEGFR(123) or sEGFR(3) were treated
with EGF in the absence or presence of the covalent
cross linking agent DSS according to published
procedures (Lax et al., *J. Biol. Chem.* 266:13828-13833,
1991; Hurwitz et al., *J. Biol. Chem.* 266:22035-22043,

35 1991). The samples were analyzed by SDS PAGE and
stained with Coomasie-blue for visualization. sEGFR(3)
migrated in SDS gel with an apparent molecule weight of

35,000 daltons (M-monomers). After cross-linking in the
presence of EGF, a slower migrating component was
detected that corresponds to sEGFR(3) covalently
cross-linked with EGF. No evidence for cross-linked
5   dimers of the sEGFR(3)/EGF complex was observed.
sEGFR(123) migrates SDS-PAGE with an apparent molecular
weight of 75,000 daltons (M-monomers). In the presence
of both EGF and DSS an additional band of apparent
molecular weight of 170,000 daltons (D-dimers) was
10   detected, corresponding to dimers of the sEGFR(123)/EGF
complex. The effect of addition of EGF to purified
preparations of sEGFR(123) and sEGFR(3) upon their beha-
vior in a covalent cross-linking experiment was
observed. A small mobility shift upon treatment with
15   the covalent cross linking agent DSS, only in the
presence of EGF, shows that EGF binds to both fragments.
In addition, cross-linked dimers are seen in the
presence of EGF and DSS for sEGFR(123), as has been
reported for sEGFR(1234) (Lax et al., J. Biol. Chem.
20   266:13828-13833, 1991; Hurwitz et al., J. Biol. Chem.
266:22035-22043, 1991), but not for sEGFR(3). Since EGF
induces dimerization of sEGFR(123), subdomain 4 appears
not to be required for receptor dimerization. sEGFR(3)
binds specifically to EGF but does not undergo EGF
25   dependent dimerization (Kohda et al., J. Biol. Chem.
268:1976-1981, 1993).

        Binding studies performed with sEGFR(123) were
consistent with a similar mode of EGF binding to that
observed with sEGFR(1234). However, low levels of
30   sEGFR(123) expression in CHO or insect cells precluded
further detailed examination. ITC studies of EGF bind-
ing to sEGFR(3) gave K_D values (Table 2) that were simi-
lar to those obtained for the second EGF binding event
to sEGFR(1234). However, the characteristics of the
35   titrations were very different. The binding isotherms
obtained from EGF/sEGFR(3) titrations were more similar
to those seen for the aFGF/sFGFR titration. EGF binds

79

to sEGFR(3) in a simple 1:1 fashion, with a K_D of 416 nM, and a negative associated enthalpy (ΔH = -2 kcal/mol). These characteristics, together with the absence of EGF-induced dimerization of sEGFR(3) are consistent with

5   domain 3 representing one half-site for EGF binding to the EGF receptor.

Other embodiments are within the following claims.

The SH2 domain protein GRB-7 is co-amplified, overexpressed and in a tight complex with HER2 in breast cancer

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SH2 domain proteins are important components of the
signal transduction pathways activated by growth factor
receptor tyrosine kinases. We have been cloning SH2
domain proteins by bacterial expression cloning using the
tyrosine phosphorylated C-terminus of the epidermal
growth factor receptor as a probe. One of these newly
cloned SH2 domain proteins, GRB-7, was mapped on
mouse chromosome 11 to a region which also contains
the tyrosine kinase receptor, HER2/erbB-2. The
analogous chromosomal locus in man is often amplified
in human breast cancer leading to overexpression of
HER2. We find that GRB-7 is amplified in concert with
HER2 in several breast cancer cell lines and that GRB-7
is overexpressed in both cell lines and breast tumors.
GRB-7, through its SH2 domain, binds tightly to HER2
such that a large fraction of the tyrosine phosphorylated
HER2 in SKBR-3 cells is bound to GRB-7. GRB-7 can
also bind tyrosine phosphorylated SHC, albeit at a lower
affinity than GRB2 binds SHC. We also find that GRB-7
has a strong similarity over >300 amino acids to a newly
identified gene in Caenorhabditis elegans. This region of
similarity, which lies outside the SH2 domain, also
contains a pleckstrin homology domain. The presence of
evolutionarily conserved domains indicates that GRB-7
is likely to perform a basic signaling function. The fact
that GRB-7 and HER2 are both overexpressed and bound
tightly together suggests that this basic signaling pathway
is greatly amplified in certain breast cancers.
Key words: C.elegans/erbB-2/pleckstrin domain/SH2
domain/tyrosine kinase

Introduction

Many growth factors bind to receptors with intrinsic tyrosine
kinase activity (Schlessinger and Ullrich, 1992; Fantl et al.,
1993). These receptors are crucial for normal development
but can also act as oncogenes leading to cell transformation.
The family of epidermal growth factor receptor (EGF-
receptor) related tyrosine kinases illustrates the diverse
functions of these receptors. One of the EGF-receptor related
tyrosine kinases, the C.elegans protein Let-23, is crucial for

proper development of the nematode vulva (Aroian et al.,
1990). Other members of this receptor family are more
recognized for their role in oncogenesis. The avian
oncogene, v-erbB, represents a truncated homologue of the
mammalian EGF-receptor (Downward et al., 1984). Another
receptor with close similarity to EGF-receptor is HER2, also
known as c-erbB-2 (Coussens et al., 1985). This receptor
was also isolated as the rat oncogene neu, an oncogene
responsible for chemically induced rat glioblastomas
(Bargmann et al., 1986). HER2/erbB-2 is known to be
amplified and overexpressed in ~25% of human breast
cancers (Slamon et al., 1987,1989). In many, but not all
studies, this HER2 overexpression correlates with a poor
prognosis (Elledge et al., 1992).

To understand the mechanism by which these receptor
tyrosine kinases are responsible for both normal development
and transformation, it is important to understand the basic
intracellular signaling mechanisms activated by these tyrosine
kinases. Recent studies have indicated an important role for
proteins with SH2 domains in growth factor receptor signal
transduction (Margolis, 1992; Pawson and Schlessinger,
1993). These proteins bind to the tyrosine phosphorylated
growth factor receptors due to a direct interaction between
the SH2 domain and the phosphotyrosine-containing peptide
sequences within the receptor (Moran et al., 1990; Margolis
et al., 1990a). The SH2 domain proteins then trigger several
different signaling cascades. Some SH2 domain proteins,
such as PLC-γ, have intrinsic enzymatic activity. The
binding of PLC-γ to the growth factor receptor tyrosine
kinase triggers the phosphorylation of this enzyme leading
to a breakdown of polyphosphoinositides (Rhee and Choi,
1992). Other SH2 domain proteins, such as phosphatidyl-
inositol-3 kinase (PI-3 kinase) associated p85, act as adaptors
coupling growth factor receptors to other signaling
molecules. As an example, p85 serves as a bridge binding
growth factor receptors to the 110 kDa PI-3 kinase (Hiles
et al., 1992).

Several SH2 domain proteins were isolated in our
laboratory by screening bacterial expression libraries with
the tyrosine phosphorylated C-terminus of the EGF-receptor
(Skolnik et al., 1991; Lowenstein et al., 1992; Margolis et
al., 1992). We called this method CORT for cloning of
receptor targets. The proteins cloned were called GRBs
(growth factor receptor bound). GRB1 was identified as the
PI-3 kinase-associated p85 (Skolnik et al., 1991). The second
protein isolated using this method was termed GRB2 and
consisted of one SH2 domain sandwiched between two SH3
domains (Lowenstein et al., 1992). GRB2 is a homologue
of the Caenorhabditis elegans protein sem-5 (Clark et al.,
1992). Sem-5 is downstream of the receptor tyrosine kinase
Let-23 and like Let-23 is crucial for vulval development.
Similarly, a Drosophila melanogaster homologue of GRB2
and sem-5, Drk, is necessary for proper eye development
where it lies downstream of the sevenless tyrosine kinase

Table 1. List of loci mapped in interspecific backcross animals

Locus	Probe name	Restriction enzyme	Restriction fragment sizes	
			AEJ/Gn fragment sizes (kb)	M.sprenus fragment sizes (kb)[a]
Ngfr	p5b	PstI	4.0, 2.9, 2.4, 1.5, 1.2	5.5, 2.4, 1.9, 1.5, 1.2
Grb-7		TaqI	2.6	3.8
Erbb-2	nevc(t)/sp6J00	PstI	3.1	4.4
Csfg	pBRG-4	TaqI	3.0	2.5
D11Mit10[b]			0.096	0.126

[a]Bold restriction fragment size indicates the segregating allele(s) that was typed in the backcross.
[b]Locus typed by PCR amplification of microsatellite sequences.

receptor (Simon et al., 1993; Olivier et al., 1993). Through a combination of biochemical and genetic studies it has been determined that GRB2 serves as a crucial link between receptor tyrosine kinases and ras. GRB2 acts as an adaptor forming a complex between activated receptor tyrosine kinases and son of sevenless (SOS), a ras GTP/GDP exchange protein (Buday and Downward, 1993; Chardin et al., 1993; Egan et al., 1993; Gale et al., 1993; Li et al., 1993, Olivier et al., 1993; Simon et al., 1993).

Recently, using CORT, we identified another novel SH2 domain protein called GRB-7 (Margolis et al., 1992). GRB-7 has a single SH2 domain at its C-terminus, a central region with similarity to ras GAP and a proline rich N-terminus. In this paper, we report that GRB-7 maps to a region on mouse chromosome 11 containing the gene for the HER2/erbB-2 receptor tyrosine kinase. This region of mouse chromosome 11 is syntenic to an area of human chromosome 17q that is often amplified in breast cancer (Slamon et al., 1987, van de Vijver et al., 1987; Buchberg et al., 1989). Accordingly, we examined GRB-7 expression in breast cancer. We find that GRB-7 is amplified and overexpressed in breast cancer in concert with the HER2 receptor. GRB-7 is bound tightly to HER2 via its SH2 domain such that the majority of tyrosine phosphorylated HER2 is complexed with GRB-7. Finally, we demonstrate that GRB-7 contains a region of > 300 amino acids with similarity to a C.elegans gene recently identified by the C.elegans genome project (Sulston et al., 1992). This region lies outside the SH2 domain and is likely crucial in GRB-7 signal transduction.

Results

GRB-7 localizes to mouse chromosome 11 near HER2/erbB-2

To determine GRB-7 chromosomal localization, liver genomic DNA from inbred and recombinant inbred (RI) strains of mice was digested with TaqI and typed by Southern blotting as described previously (Sap et al., 1990). This analysis revealed two allelic forms of GRB-7, whose inheritance in RI strains of mice defined a single locus. Comparison of the strain distribution pattern for GRB-7 with those for 1124 other markers distributed over all of the mouse chromosomes allowed GRB-7 to be localized to chromosome 11 on the basis of its tight linkage to markers such as HoxB (one recombinant among 31 informative RI strains scored), Gfap (five recombinants among 52 strains) and Kn-1 (one recombinant among 31 strains).

To refine the localization of GRB-7, we determined its

A

Ngfr
Grb-7, Erbb-2, Csfg
D11Mit10
89 91 2 3 5 1

B

17q21-q22 — Ngfr
　　2.6
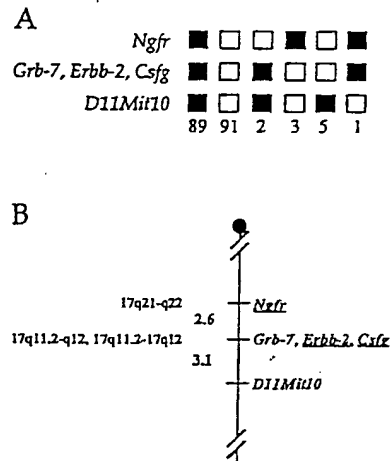17q11.2-q12, 17q11.2-17q12 — Grb-7, Erbb-2, Csfg
　　3.1
— D11Mit10

Fig. 1. GRB-7 maps on chromosome 11 near to HER2/erbB-2. (A) Summary of the results of the interspecific backcross analysis. Genes mapped in the analysis are listed on the left. Each column represents the chromosome identified in the N2 progeny inherited from the (AEJ/GN×M.sprenus) F1 parent. The closed boxes represent the AEJ/GN allele and the open boxes represent the M.sprenus allele. The numbers of each type of chromosome identified in the backcross progeny are listed at the bottom. (B) Genetic localization of GRB-7. The figure represents the region of mouse chromosome 11 analyzed in the interspecific backcross. The genes mapped are listed on the right and the genetic map distance (in cM) between adjacent loci, are listed on the left of the chromosome. The location of the human homologues of the genes are listed on the extreme left.

location on mouse chromosome 11 in an interspecific backcross, that has been typed for > 30 genetic markers distributed throughout its length. Genomic DNAs from AEJ/Gn and Mus sprenus parental control animals were digested with several restriction endonucleases and analyzed by Southern blot hybridization using probes that identified the following loci: Ngfr, Grb-7, Erbb-2 and Csfg (Table I). At least one restriction fragment length polymorphism (RFLP) was identified for each of the probes tested and the sizes of the genomic restriction fragments detected by each
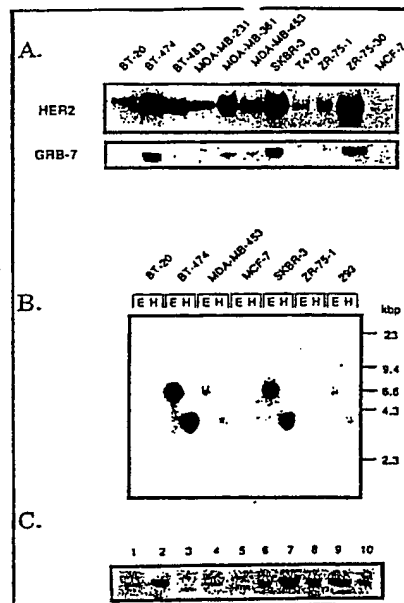
Fig. 2. GRB-7 is amplified and overexpressed in breast cancer cell lines. (A) Immunoblotting of GRB-7 in breast cancer cell lines. Protein (200 µg) from cell lysates was run on an SDS gel and transferred to nitrocellulose. The nitrocellulose was then blocked and probed with either anti-GRB-7 (#188) antibody or anti-HER2 antibody. After detection with [125I]protein A, blots were exposed to film. The HER2 blot was exposed for 4 h and the GRB-7 blot for 12 h. (B) Southern blotting of GRB-7 in breast cancer cell lines. Genomic DNA (10 µg) was digested with either EcoRI (E) or HindIII (H) and separated on a 0.7% agarose gel. All lanes represent breast cancer cell lines except 293 cells (human embryonic kidney), which were used as a control. Blotting and hybridization were carried out with a GRB-7 probe as described in Materials and methods. (C) Immunoblotting of GRB-7 in breast tumors. Frozen pulverized tumor powder was lysed in 5% SDS as described (Tandon et al., 1989) and 100 µg of total solubilized protein was run on SDS[EN]PAGE, transferred to nitrocellulose and immunoblotted with anti-GRB-7 (#188) antibody. After detection with [125I]anti-rabbit sera (Amersham) the nitrocellulose blots were exposed to film. A representative blot of 10 tumors is shown. All tumors overexpressed HER2 except #5. With the exception of #3 and #5, all tumors were considered positive for GRB-7 expression.

probe are listed in Table I. To determine the size polymorphism for D11Mit10, parental DNAs and F1 controls were polymerase chain reaction (PCR) amplified using the oligomers defining D11Mit10. As expected, a simple sequence length polymorphism (SSLP) was detected between AEJ/Gn and M.spretus (Table I).

The segregation pattern of the M.spretus allele in 191 backcross animals was then determined for Ngfr, Grb-7, Erbb-2 and Csfg (by Southern blot hybridization) and for D11Mit10 by PCR amplification and agarose gel electrophoresis. The results are summarized in Figure 1.

Mice were either homozygous for the AEJ/Gn allele or heterozygous for the M.spretus and AEJ/Gn alleles. The frequency of AEJ/Gn and M.spretus alleles for the loci mapped in the N2 progeny did not significantly differ from the expected 1:1 ratio. The results presented here are consistent with the previous genetic localization of Ngfr, Erbb-2 and Csfg using a [(C57BL/6J × M.spretus) × C57BL/6J interspecific backcross (Buchberg et al., 1989). Gene order was determined by minimizing the number of multiple recombinants between loci. The order of the loci and the ratio of the number of recombinants to the total number of N2 offspring examined for each locus are: Ngfr − 5/191 − (Grb-7, Erbb-2, Csfg) − 6/191 − D11Mit10. The genetic distances between the loci in centimorgans ± standard error are: Ngfr − 2.6 ± 1.1 − (Grb-7, Erbb-2, Csfg) − 3.1 ± 1.2 − D11Mit10. No recombinants were detected between Grb-7, Erbb-2 and Csfg in 191 N2 progeny, indicating that these loci are tightly linked and must lie < 1.6 cM apart (upper 95% confidence limit).

GRB-7 is amplified and overexpressed in breast cancer cell lines

Next, we determined if GRB-7 is amplified and overexpressed with HER2 in breast cancer. We checked the expression of GRB-7 in several breast cancer cell lines by immunoblotting cell lysates for GRB-7 expression. We found a close correlation between HER2 and GRB-7 expression (Figure 2A). To confirm that this overexpression was due to gene amplification, we performed Southern blotting with a GRB-7 probe (Figure 2B). GRB-7 is amplified in both SKBR-3 cells and BT-474 — two cell lines in which HER2 is also amplified (Kury et al., 1990). Neither GRB-7 nor HER2 is amplified in the other cell lines.

GRB-7 is overexpressed in breast cancer tissue

Immunoblotting with the GRB-7 antibody was then performed on 72 breast cancer samples in which HER2 expression had previously been analyzed, also by an immunoblot procedure (Ciocca et al., 1992). A representative result for 10 such tumors is displayed in Figure 2C. Thirty-four of the breast cancer specimens exhibited HER2 overexpression, and 24 of these were concomitantly positive for GRB-7 expression (Table II). In the 38 tumors negative for HER2 overexpression, only eight were positive for GRB-7. These results were statistically significant by chi-square analysis (P < 0.001), and they confirm the cell line data, suggesting that GRB-7 is often expressed in human breast tumors in concert with HER2 overexpression.

GRB-7 binds tightly to HER2

We next examined whether the GRB-7 protein was physically associated with the HER2 receptor. This might be expected
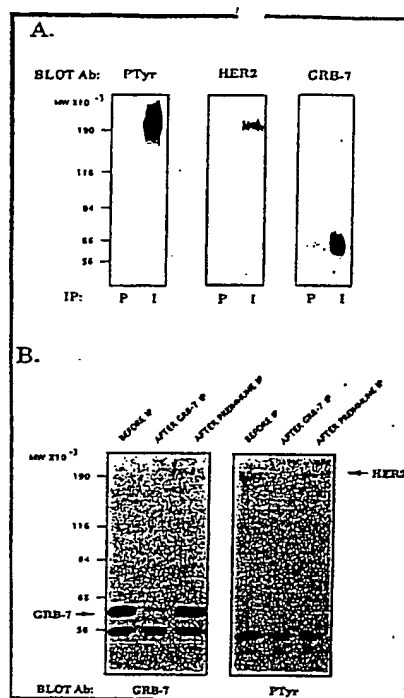
83

Fig. 3. GRB-7 is tightly bound to HER2. (A) Coimmunoprecipitation of HER2 and GRB-7. SKBR-3 cells were starved overnight in serum free medium and then lysed in 1% Triton X-100 lysis buffer with phosphatase and protease inhibitors. Lysates were then immunoprecipitated with anti-GRB-7 (I) or pre-immune (P) serum and separated by SDS[EN]PAGE. After transfer to nitrocellulose, blots were probed with phosphotyrosine (PTyr), HER2 or GRB-7 (#188) antibodies. Blots were detected as in Figure 2. HER2 and PTyr blots represent immunoprecipitations from 2.5 mg of cellular protein while the GRB-7 blot is an immunoprecipitation from 1 mg of protein. GRB-7 and PTyr blots were exposed for 14 h while the HER2 blot was exposed for 4 h. (B) GRB-7 immunoprecipitation clears tyrosine phosphorylated HER2 from SKBR-3 cell lysates. Cell lysate (500 mg) from starved SKBR-3 cells was immunoprecipitated with 20 µl of pre-immune or anti-GRB-7 (#212) serum. Lysates (130 µg) from before and after the immunoprecipitations were then immunoblotted with anti-PTyr and anti-GRB-7 (#188) to determine what fraction of GRB-7 and tyrosine phosphorylated HER2 was cleared from the lysate by immunoprecipitation. The band at 56 kDa in the GRB-7 blot is an unidentified protein recognized by affinity purified antibody #188. It is not recognized by any of the other GRB-7 antibodies in immunoprecipitation or immunoblotting. Exposure time was 14 h.

as GRB-7 was cloned based on its ability to bind to the EGF-receptor, a receptor closely related to HER2 (Coussens et al., 1985). To perform these studies, GRB-7 was immuno-precipitated from serum-starved SKBR-3 cells and then blotted with phosphotyrosine or HER2 antibodies. Antibodies to GRB-7 specifically immunoprecipitated a

phosphotyrosine-containing band at 190 kDa (Figure 3A, left panel) which was identified as HER2 (Figure 3A, middle panel). This co-immunoprecipitation was not seen with pre-immune serum. No other bands were detected in the antiphosphotyrosine blot, indicating that GRB-7 was not tyrosine phosphorylated (Figure 3A, middle and right panels). This co-immunoprecipitation between GRB-7 and HER2 was demonstrated with three different GRB-7 antisera, indicating it was not dependent on the antibody used. The co-immunoprecipitation of GRB-7 and HER2 was also seen in BT474 cells (results not shown).

To determine what percentage of HER2 was bound to GRB-7 in these cells, we immunoprecipitated GRB-7 from a small number of cells such that we depleted the lysate of GRB-7. Under these conditions, the lysates were almost completely cleared of tyrosine phosphorylated HER2 as well as GRB-7 (Figure 3B). Pre-immune serum which did not immunoprecipitate GRB-7 did not significantly affect the tyrosine phosphorylated HER2 in the lysates. It should be noted that GRB-7 immunoprecipitation did not measurably affect the total amount of HER2 in the cell lysate as only a small fraction of HER2 is tyrosine phosphorylated and able to bind GRB-7. These results indicate a strong association between tyrosine phosphorylated HER2 and GRB-7.

GRB-7 binds SHC

We next asked whether GRB-7 became tyrosine phos-phorylated after ligand stimulation of growth factor receptors. Because the true ligand or ligands for HER2 is still unclear (Peles et al., 1993; Plowman et al., 1993), we stimulated tyrosine phosphorylation by activation of the EGF-receptor in SKBR-3 cells (King et al., 1988). After stimulation with EGF, several additional bands become tyrosine phosphorylated yet tyrosine phosphorylation of GRB-7 was still undetectable (Figure 4). Phosphoamino acid analysis of GRB-7 before and after stimulation revealed the presence of phosphoserine and phosphothreonine but no phosphotyrosine (results not shown).

We did find that GRB-7 antibodies immunoprecipitated a tyrosine phosphorylated protein of 54 kDa after EGF stimulation (Figure 4). We suspected that this 54 kDa band might be tyrosine phosphorylated SHC (Pelicci et al., 1992) as it has been found that another SH2 domain protein, GRB2, binds tightly to phosphorylated SHC (Rozakis-Adcock et al., 1992; Skolnik et al., 1993). To confirm this suspicion, we immunoprecipitated GRB-7 from EGF-stimulated SKBR-3 cells and immunoblotted with anti-SHC antibodies (Figure 4, right panel). We were able to detect EGF-stimulated association of SHC and GRB-7.

We next wanted to determine whether GRB-7 binds directly to HER2 and SHC through the GRB-7 SH2 domain. The association of the GRB2 SH2 domain with SHC presumably occurs due to the tyrosine phosphorylation of SHC at residue 317 (Rozakis-Adcock et al., 1992; Skolnik et al., 1993, Songyang et al., 1993). The sequence around this tyrosine, YVN, is felt to represent a high affinity binding site for the SH2 domain of GRB2 when tyrosine phosphorylated. A similar motif is present in EGF-receptor at Tyr1068 (YIN) and in HER2 at Tyr1139 (YVN). We suspected that the GRB-7 SH2 domain might also bind to both HER2 and SHC via this motif. To study this problem, we prepared a GST fusion protein of the GRB-7 SH2 domain containing a protein kinase A phosphorylation site (Ron and
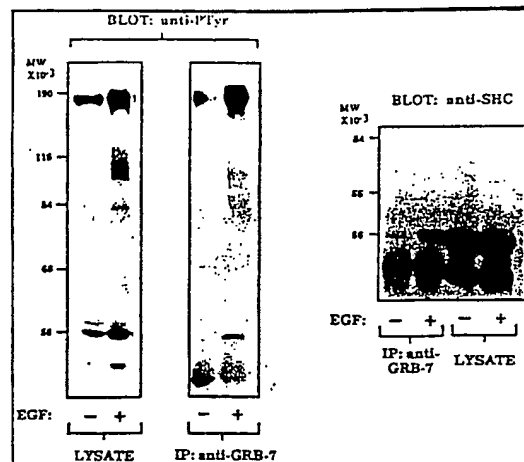
Fig. 4. GRB-7 antisera co-immunoprecipitates SHC from EGF-stimulated SKBR-3 cell lysates. SKBR-3 cells were starved in serum free media overnight and then treated with or without 200 nM EGF for 3 min. After cell lysis, immunoprecipitation was performed with anti-GRB-7 (#222) or anti-SHC antibodies on 1.5 mg of cell lysate. Immunoprecipitates and cell lysate (130 μg) were then run on SDS[EN]PAGE and transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (PTyr), or anti-SHC antibodies. In the SHC immunoblot, the band at ~50 kDa represents the IgG heavy chain in the GRB-7 immunoprecipitate while in the cell lysate it represents the 46 kDa form of SHC.

Dressler, 1992). Phosphorylation by protein kinase A in the presence of [γ-32P]ATP was then used to label the GST fusion protein. SKBR-3 lysates were separated by SDS[EN]PAGE, transferred to nitrocellulose and probed with the labeled GRB-7 SH2 domain. During these incubations, we added a 100-fold molar excess of unlabeled GST, GST−GRB-7-SH2 or GST−GRB2. With GST alone, the GRB-7 SH2 domain bound to two major bands at 190 kDa and 54 kDa, corresponding to HER2 and SHC (Figure 5A, left panel). This demonstrates that GRB-7 can bind directly to these proteins in cells and does not require intermediate molecules. As expected, unlabeled GRB-7 competed the binding to both proteins (Figure 5A, middle panel). We found that GRB2 could prevent the GRB-7 SH2 domain from binding to SHC but did not affect HER2 binding (Figure 5A, right panel).

When we probed the same lysates with labeled GRB2 at a similar concentration and specific activity as the GRB-7-SH2 domain, GRB2 showed strong binding to SHC but no binding to HER2 (Figure 5B). We did detect binding of GRB2 to a protein at 160 kDa which is not HER2 but likely represents the association of the GRB2 SH3 domains to SOS (Buday and Downward, 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993). Unlabeled GRB-7 could not compete the binding of GRB2 to SHC in contrast to the fact that GRB2 could easily compete GRB-7 binding to SHC. Taken together, these results indicate that GRB-7 binds SHC probably at the same Y(V/I)N motif as GRB2. However, the affinity of GRB2 for SHC appears much greater than that of GRB-7. In contrast, GRB-7 binds tightly to HER2, but GRB2 does not compete this binding.

GRB-7 is phosphorylated by the EGFR−HER2 chimera
To determine if GRB-7 was tyrosine phosphorylated by activated HER2, we transfected GRB-7 into NIH 3T3 cells containing the chimeric EGFR−HER2 receptor (Lee et al., 1989). These cells contain the HER2 intracellular domain fused to the EGF-receptor extracellular domain such that EGF can stimulate the HER2 tyrosine kinase. After EGF stimulation of these cells, GRB-7 becomes tyrosine phosphorylated and associates with the chimeric receptor in a ligand dependent fashion (Figure 6A). EGF also induces association between GRB-7 and an unidentified tyrosine phosphorylated protein of 70 kDa. We examined the binding of the [32P]GRB-7 SH2 domain to lysates from NIH 3T3 cells expressing the EGFR−HER2 chimera (Figure 6B). We detected binding to three bands: the chimeric receptor at 190 kDa, the 54 kDa SHC band and the unidentified band at 70 kDa. The binding of the GRB-7 SH2 domain to these proteins was enhanced in lysates from EGF-stimulated cells. There was some binding of the probe to the receptor even in the absence of ligand because the receptor is autophosphorylated to some extent in the untreated cells. GRB2 was able to completely compete the binding of the GRB-7 SH2 domain to SHC but did not significantly affect the binding to the 70 kDa protein. GRB2 weakly competed with GRB-7 for binding to the chimeric receptor.

Primary sequence of GRB-7 reveals homology to a putative gene in C.elegans and the pleckstrin domain
Our data indicate that GRB-7 is a signaling partner for HER2 especially in breast cancer cells where both are overexpressed. However, it is not clear what signal might
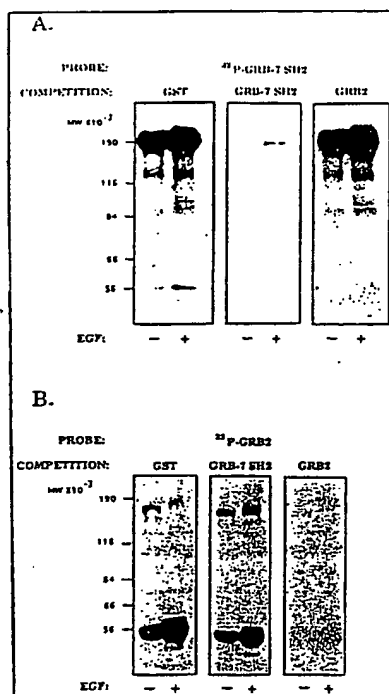
85

Fig. 5. Binding of GRB-7 SH2 domain and GRB2 to SKBR-3 lysates. GST fusion proteins of GRB-7 SH2 domain and full-length GRB2 were prepared in a GST vector that incorporates a protein kinase A phosphorylation site. The fusion proteins were labeled using protein kinase A and [γ-32P]ATP as described in Materials and methods. SKBR-3 lysates, treated with or without EGF were then separated by SDS[EN]PAGE and transferred to nitrocellulose. These blots were blocked for 2 h at room temperature in 5% non-fat milk and incubated with 12.5 ng/ml (specific activity 2×10⁷ c.p.m./μg) of probe. Included with the probe was a 100-fold molar excess of either GST, GRB-7 SH2 or GRB2 protein. (A) GRB-7 SH2 domain probe, exposure time 12 h. (B) GRB2 probe, exposure time 2 h.

be sent through GRB-7 when it binds and perhaps becomes phosphorylated by HER2. We have recently detected a close similarity between GRB-7 and a putative gene identified by the *C.elegans* genome sequencing project (Sulston *et al.*, 1992). This putative *C.elegans* gene, F10E9.6, encodes a predicted protein of 650 amino acids with no SH2 domain (Figure 7A). The region of similarity spans ~330 amino acids with an identity of 28% and similarity of 38% (Figure 7B). Using the randomization utility of the alignment program, Bestfit (Devereux *et al.*, 1984), we found that the optimal alignment score for GRB-7 to F10E9.6 lies >18 standard deviations from the mean of scores from 10

scrambled alignments. This indicates a highly significant relationship between the two proteins.

This region of similarity between F10E9.6 and GRB-7 also contains a pleckstrin domain. First described by Mayer and coworkers (Mayer *et al.*, 1993) as well as Haslam and coworkers (Haslam *et al.*, 1993), the pleckstrin domain is found in several different signaling molecules including pleckstrin (Tyers *et al.*, 1988), the serine kinase akt/rac (Bellacosa *et al.*, 1991; Jones *et al.*, 1991) ras GAP (Trahey *et al.*, 1988; Vogel *et al.*, 1988) and the SH3 binding protein, 3BP-2 (Ren *et al.*, 1993). In fact, the similarity we initially detected between ras GAP and GRB-7 encompasses the pleckstrin domain (Margolis *et al.*, 1992). It has been suggested that the pleckstrin motif may function as a protein binding domain (Haslam *et al.*, 1993; Mayer *et al.*, 1993; Musacchio *et al.*, 1993). An alignment of GRB-7, F10E9.6 and the derived consensus sequences for the pleckstrin domains is shown in Figure 7C. A more detailed alignment of these proteins in the context of other pleckstrin domain proteins has also recently been published (Musacchio *et al.*, 1993). While it is not known what signal is relayed by GRB-7, it is clearly a protein composed of evolutionarily conserved domains, indicating that it likely performs a basic signaling function.

Discussion

In this paper we report an amplification and overexpression of GRB-7 in several different breast cancer cell lines. GRB-7 overexpression is also found in tissue samples from primary human breast cancer. This overexpression correlates with HER2 expression indicating that these two genes are often co-amplified and/or co-expressed. Other genes on human chromosome 17q have also been found to be co-amplified with HER2. Although c-erbA –thyroid hormone receptor is co-amplified with HER2, overexpression of this protein has not been detected (van de Vijver *et al.*, 1987; Tavassoli *et al.*, 1989). Topoisomerase IIa has been found to be co-amplified with HER2 but only in a relatively small percentage of the cases (Smith *et al.*, 1993). Our results indicate that GRB-7 is likely present in an amplicon which also contains HER2 and which represents a region on 17q. This region on 17q lies proximal to the locus containing the breast cancer susceptibility gene, BRCA1 (Bowcock *et al.*, 1993).

Not only are GRB-7 and HER2 co-expressed in breast cancer but they also exist in a tight complex. In SKBR-3 cells, we co-immunoprecipitate a large fraction of the tyrosine phosphorylated HER2 with GRB-7 antibodies. The fraction of total HER2 that is bound to GRB-7 is relatively low, as only a small percentage of HER2 is tyrosine phosphorylated in these unstimulated cells. GRB-7 is not only bound to HER2 in SKBR-3 cells but can also bind SHC. The binding of GRB-7 to SHC gives us some insight into the tyrosine phosphorylation sites to which the GRB-7 SH2 domain can bind. It seems likely that the GRB-7 SH2 domain binds the Y(V/I)N motif on SHC with a lower affinity than GRB2. GRB2, which competes with GRB-7 for binding to SHC does not affect the binding of GRB-7 to HER2 in the SKBR-3 cells. This suggests that GRB-7 either binds to a site other than Y(V/I)N on HER2 or binds to the Y(V/I)N site on HER2 but with a much greater affinity than GRB2. It should be noted that GRB2 does not bind HER2 from the

Fig. 6. GRB-7 phosphorylation and binding in NIH 3T3 cells expressing the EGFR−HER2 chimera. (A) Tyrosine phosphorylation of GRB-7. Cells expressing the EGFR−HER2 chimera with and without GRB-7 were starved in serum free media for 48 h and then treated with EGF for 3 min. Lysates (1.5 mg protein) were immunoprecipitated with GRB-7 antibodies and separated by SDS[EN]PAGE. After transfer to nitrocellulose, blots were probed with anti-PTyr or anti-GRB-7 antibodies. Eighty percent of the immunoprecipitate was run for anti-PTyr blot and 20% for GRB-7 blot. (B) GRB-7 SH2 domain associates with three proteins in NIH 3T3 cells expressing the EGFR−HER2 chimera. Lysates (50 μg) from cells expressing the chimeric receptor were run out on SDS[EN]PAGE and transferred to nitrocellulose. Blots were then probed with [32P]GRB-7 SH2 domain as in Figure 5.

Fig. 7. Comparison of GRB-7 with the putative C.elegans gene F10E9.6 and the consensus sequence for the pleckstrin domain. (A) Schematic representation of GRB-7 and F10E9.6. F10E9.6 represents a putative gene derived from genomic sequence of C.elegans using the program Genefinder. The sequences were deposited by the C.elegans Sequencing Consortium, Genbank accession number L10986 (Sulston et al., 1992). (B) Alignment of the region of similarity between GRB-7 and F10E9.6. Alignment was performed using the GCG Bestfit program with bold capital letters indicating identity and plain capital letters indicating conservative substitution as defined by a score > 0.8 on the PAM 250 scoring table (Schwartz and Dayhoff, 1979). (C) Alignment of GRB-7 and F10E9.6 with the consensus sequences for pleckstrin domain. Bold capital letters indicate agreement with the consensus sequence as defined by both Mayer (Mayer et al., 1993) and Haslam (Haslam et al., 1993), while plain capital letters indicate agreement with only one of the consensus sequences. ♦ represents hydrophobic residues and ✦ represents aromatic residues.

SKBR-3 cells yet binds to the EGFR—HER2 chimeric receptor after EGF stimulation (J.W. and B.M., unpublished results). This suggests that the intensity or sites of autophosphorylation on the EGFR—HER2 chimera may be different than on HER2 from SKBR-3 cells.

The tyrosine phosphorylation sites of HER2 and EGF-receptor are closely related in sequence (Hazan et al., 1990; Segatto et al., 1990). Thus it is likely that GRB-7 binds to the same site on HER2 and EGFR. However, attempts to inhibit the binding of GRB-7 to HER2 using tyrosine phosphorylated peptides from the EGF-receptor have been to date, unsuccessful. It has been notoriously difficult to map SH2 domain binding sites on the EGF-receptor in the past, due to the close proximity and overlapping binding of several of the autophosphorylation sites. For example, PLC-g binds with high affinity to three different autophosphorylation sites (Rotin et al., 1992). Thus further work will be necessary to determine the actual binding preference of the GRB-7 SH2 domain. Other clues to the exact binding site of GRB-7 may come from the identity of the 70 kDa protein which binds to the SH2 domain of GRB-7 in the EGF-stimulated NIH 3T3 cells expressing the EGFR—HER2 chimera. Our data suggest that this protein is not the 66 kDa form of SHC because GRB2 cannot compete this binding. Additionally, we could not detect the 66 kDa form of SHC in GRB-7 immunoprecipitates (J.Wu and B.M., unpublished observations). Other potential candidates for this protein are PTP-1D/Syp (Feng et al., 1993; Vogel et al., 1993) or paxillin (Birge et al., 1993).

In contrast to the results obtained in the SKBR-3 cells, GRB-7 was tyrosine phosphorylated in EGF-stimulated cells expressing chimeric EGFR—HER2 receptor. The phosphorylation state of GRB-7 in breast tumors in vivo remains to be determined. Nonetheless, there is a distinct possibility that the binding of GRB-7 to the receptor may be sufficient to initiate the GRB-7 signaling pathway. Several proteins such as GRB2 and PI-3 kinase-associated p85 appear to transduce their signal without tyrosine phosphorylation (Backer et al., 1992; Lowenstein et al., 1992; Carpenter et al., 1993). For these adaptor proteins, the binding of the SH2 domain to the receptor appears sufficient to initiate the signaling cascade.

One of the major questions that remains is the signal relayed by GRB-7. The SH2 domain proteins are grouped into two major classes: those that have intrinsic catalytic or signaling activity and those that act as adaptors coupling secondary proteins to growth factor receptor tyrosine kinases (Margolis, 1992; Pawson and Schlessinger, 1993). The one clue that GRB-7 may act as an adaptor molecule is the fact that GRB-7 contains a sequence similar to the recently described pleckstrin domain. This motif is found in several proteins that already have catalytic activity (Haslam et al., 1993; Mayer et al., 1993; Musacchio et al., 1993). In these proteins, it is theorized that the pleckstrin domain has a role in binding other regulatory factors. One possible example of this function is the association of the pleckstrin domain on the β-adrenergic receptor kinase with βγ subunits of heterotrimeric G-proteins (Koch et al., 1993; Musacchio et al., 1993). The amino acid sequence identity between different pleckstrin domains is <20%, so more studies will be required before a uniform function can be assigned to this domain.

The alignment between the putative C.elegans gene,

F10E9.6. and GRB-7 is more significant and the two genes likely function in a similar fashion. The F10E9.6 gene is theorized to exist based on the analysis of the C.elegans genomic sequence using the program Genefinder (Sulston et al., 1992). This similarity includes the pleckstrin domain and a second region which extends toward the N-terminus. It is also interesting to note that GRB-7 and F10E9.6 both have proline rich domains but that F10E9.6 does not appear to have a SH2 domain. Such proline rich regions are potential binding sites for SH3 domains (Li et al., 1993; Ren et al., 1993). One gene that has been mapped to the same region of the C.elegans genome as F10E9.6 is mig-10. mig-10 was isolated as a gene involved in longitudinal neuronal migration in the nematode (Manser and Wood, 1990). Certain cells in C.elegans such as the canal-associated neuron (CAN) and the hermaphrodite specific neuron (HSN) must undergo long range migrations which they do not complete in the mig-10 mutants. There are > 20 genes known to be required for this process but the identity of these genes is not yet known (Wadsworth and Hedgecock, 1992). There is preliminary evidence that mig-10 could be encoded by F10E9.6 based on cosmid rescue data and partial cDNA sequence (J.Manser, personal communication). However, further studies on the relationship of F10E9.6, mig-10 and GRB-7 will be required.

Our data seem to suggest a basic signaling function for GRB-7, yet GRB-7 is expressed only in kidney, liver and gonads, suggesting it may have a more specialized function. Recently, in a CORT screen of NIH 3T3 cells using the tyrosine phosphorylated EGF-receptor, we have isolated a second gene with high similarity to GRB-7 (J.Ooi, V.Y., D.Immanuel and B.M, unpublished data). This gene, which we call GRB-10, is ~60% identical to GRB-7 through the SH2 domain and the central F10E9.6 domain. GRB-10 appears to be more widely expressed than GRB-7, indicating that the basic signaling function of GRB-7 may be performed by a family of related genes in different tissues.

One point of future interest is the effect of GRB-7 overexpression on breast cancer biology. Currently, our results indicate that GRB-7 expression alone cannot transform NIH-3T3 cells (J.W. and B.M. unpublished results). In the NIH 3T3 cells which express GRB-7 and the chimeric EGFR—HER2 receptor, our data are inconclusive because these cell lines are already transformed due to the high expression of the chimeric receptor. Whether GRB-7 expression, like HER-2, has prognostic significance in patients with primary breast cancer remains to be seen. Although our data indicate a highly significant correlation between overexpression of HER-2 and overexpression of GRB-7 in patient samples, the relationship is imperfect; 24 of the 34 specimens overexpressing HER-2 also overexpress GRB-7 but 10 do not. Similarly, about one-third of HER-2 negative tumors still overexpress GRB-7. It is interesting to speculate that those tumors overexpressing both proteins would have particularly aggressive course and worse patient outcome, a hypothesis we are now testing. Aside from their role in cancer, GRB-7 and related genes are likely to have a basic function in signal transduction. A combination of biochemical studies in mammalian cells and genetic studies in D.melanogaster and C.elegans led to the elucidation of the function of GRB2. Likewise, a similar combination of genetics and biochemical studies will lead to a better understanding of the function of GRB-7.

2187781

Materials and methods

Tissue culture

Breast cancer cell lines were originally obtained from the ATCC and grown in DMEM, 4500 mg/dl glucose with penicillin/streptomycin and 10% fetal calf serum. All NIH 3T3 cells were grown in the same media but using 10% calf serum. NIH 3T3 cells expressing the EGFR – HER2 chimera were obtained from Drs A.Zilberstein and A.Ullrich. For experiments cells were starved as described in the figure legends.

Immunoprecipitation and immunoblotting.

Several rabbit polyclonal antibodies were generated against GRB-7. Three antibodies were generated against GST fusion proteins of GRB-7 consisting of amino acids 419 – 535 (#191), amino acid 297 – 535 (#193) and the full-length protein (#222). An antipeptide antibody was generated against amino acid sequence 264 – 279 (#188). Antibodies were either used as whole serum or were affinity purified. For affinity purification, the fusion protein or peptide was immobilized on Affi-gel 10 (Biorad). Serum was concentrated by 50% ammonium sulfate precipitation and dissolved in 10 mM Tris, pH 7.5. The ammonium sulfate precipitated antibodies were then purified on the antigen column as described (Harlow and Lane, 1988), eluting the antibody with 100 mM glycine, pH 2.5. Immunoprecipitation and immunoblotting in the cell lines were performed as previously described (Margolis et al., 1989). Immunoblotting of the breast tumor samples with the GRB-7 antibody was performed using 100 µg of sodium dodecyl sulfate-solubilized protein extract as previously described (Tandon et al., 1989). SHC antibodies were purchased from Signal Transduction Labs (Lexington, Kentucky). Rabbit polyclonal antibodies directed against the C-terminus of HER2 were generously provided by Dr A.Zilberstein. Rabbit polyclonal antiphosphoryrosine antibodies were prepared using standard techniques (Kamps and Sefton, 1988).

GRB-7 constructs

GST fusion proteins were generated by PCR of GRB-7 incorporating BamHI restriction sites into the priming oligonucleotides. For the GST – GRB-7 SH2 domain probe, the sequence encoding amino acids 419 – 535 was amplified, digested with BamHI and ligated into BamHI digested pGSTag vector (Ron and Dressler, 1992). The pGSTag vector was a gift from D.Ron and the GRB2 pGSTag construct was a gift from E.Lowenstein and J.Schlessinger. For mammalian expression of GRB-7, GRB-7 was cut from the λEXlox plasmid with XbaI and MseI and blunt ended using Klenow fragment. This blunt end fragment was ligated into EcoRV digested PMJ30 vector (Margolis et al., 1990b). NIH 3T3 cells were transfected as previously described (Margolis et al., 1990b) using GRB-7 in the antisense direction as a control. The hygromycin-B phosphotransferase gene was cotransfected with the GRB-7 constructs as a selectable marker.

GST blotting

The pGSTag constructs were labeled with [γ-32P]ATP as described using 0.2 U/µl protein kinase A (Ron and Dressler, 1992). Blots were incubated for 2 h at room temperature in block buffer (20 mM HEPES, pH 7.5, 5 mM MgCl₂, 1 mM KCl, 5 mM DTT, 5% non-fat dry milk and 0.02% sodium azide) and probed for 2 h at room temperature in the same buffer using 12.5 ng (3×10⁵ d.p.m.) per ml of probe. Blots were washed four times for 15 min with Tris buffered saline (10 mM Tris pH 7.5, 150 mM NaCl) containing 0.1% Triton X-100 before exposing.

Southern blotting

Genomic DNA was prepared from tissue culture cells using standard techniques (Sambrook et al.,1989). DNA (10 µg) was digested with EcoRI or HindIII and separated on 0.7% agarose gel. The DNA was transferred to Nytran (Schleicher and Schuell) using capillary action and cross-linked by UV light. The blot was blocked for 4 h at 42°C in hybridization buffer (40% formamide, 10×Denhardt's, 1% SDS, 6×SSC and 100 µg/ml salmon sperm DNA) and then incubated with probe (2×10⁶ d.p.m./ml) overnight at 42°C. The probe consisted of a PCR product from mouse GRB-7 encompassing nucleotides 1437 – 1909. The probe was labeled with [32P]dCTP using a random priming kit (USB). The blots final wash was 0.5×SSC, 0.1% SDS at 42°C.

DNA analysis

All DNA and protein database searches were performed using the University of Wisconsin Genetics Computer Group Sequence Analysis Software (GCG) package (Devereux et al., 1984). The GenEMBL database were searched using Fasta and Tfasta, respectively (Pearson and Lipman, 1983). Protein alignments were performed with the GCG program, Bestfit. Conservative substitutions were defined as a score of ≥0.8 using the scoring table of

Schwartz and Dayhoff (Schwartz and Dayhoff, 1979), as modified by Gribskov and Burgess (Gribskov et al., 1984)

Chromosome mapping

The interspecific backcross between [AEJ/Gn-a bpH/abpH × M.spretus] F1 × AEJ/Gn-a bpH/abpH was previously described (Marini et al., 1993). Genomic DNA extractions, restriction endonuclease digestions, agarose gel electrophoresis, Southern blot transfers, hybridizations and washes were as described (Ma et al., 1993). DNA oligonucleotides used for detecting the SSLP marker was made using an Applied Biosystems Model 393 DNA synthesizer. SSLP markers were detected by amplifying genomic DNA from N2 animals using the specified DNA oligonucleotide pairs (Dietrich et al., 1992) and Taq DNA polymerase as described (Ma et al., 1993). The results of the interspecific backcrosses were analyzed by calculating the maximum likelihood estimates of linkage parameters as described. (Green, 1981)

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2187781

<u>Claims</u>

What is claimed is:

    1. Method for treatment of a disease or condition in an organism, characterized by an abnormality in
5 a signal transduction pathway, wherein said signal transduction pathway involves the interaction between:
    (a) a protein with a BLM domain and a BLM domain natural binding partner; or
    (b) domain 1 and/or domain 3 of an EGF receptor and
10 an EGF ligand,
comprising the step of disrupting or promoting said interaction <u>in vivo</u>.

    2. Method for diagnosis of a disease or condition characterized by an abnormality in a signal
15 transduction pathway, wherein said signal transduction pathway involves the interaction between:
    (a) a BLM domain and a BLM natural binding partner; or
    (b) domain 1 and/or domain 3 of an EGF receptor and
20 an EGF ligand,
comprising the step of detecting the level of said interaction as an indication of said disease or condition.

    3. Method for screening for an agent useful
25 for treatment of a disease or condition characterized by an abnormality in a signal transduction pathway, wherein said signal transduction pathway involves the interaction between:
    (a) a BLM domain and a BLM natural binding partner;
30 or
    (b) domain 1 and/or domain 3 of an EGF receptor and an EGF ligand,

comprising assaying potential said agents for those able
to disrupt or promote said interaction as an indication
of a useful said agent.

       4.   The method of any of claims 1-3 wherein
5 said disease or condition is selected from the group
consisting of cell adhesion diseases, neuronal diseases,
diseases exhibiting a defect in cell movement, breast
cancer, diseases associated with abnormal cell
development, atherosclerosis, platelet, mononuclear, and
10 neutrophil-associated diseases, and inflammation.

       5.   The method of any of claims 1-3 wherein
said disease or condition is further characterized by
abnormal cell proliferation.

       6.   The method of claim 5, wherein said
15 disease or condition is selected from the group
consisting of cancer and psioriasis.

       7.   The method of claim 3 wherein said agent
is not an antibody.

       8.   The method of claim 3 wherein said agent
20 consists essentially of domain 1 and/or domain 3 of said
EGF receptor.

       9.   The method of claim 3 wherein said agent
consists essentially of an EGF ligand fragment that
binds domain 1 and/or domain 3 of said EGF receptor.

25   10. Isolated, purified, or enriched peptide
consisting of a BLM domain.

       11. The peptide of claim 10, wherein said
peptide is produced recombinantly.

12. The peptide of claim 10, wherein said peptide is purified.

13. A composition comprising the peptide of claim 10 and a pharmaceutically acceptable carrier or
5 diluent.

14. The peptide of claim 10, wherein said BLM domain consists of amino acids 95 to 231 of GRB-7.

15. The peptide of claim 10 wherein said BLM domain consists of amino acids 95 to 428 of GRB-7.

10   16. The peptide of claim 10 wherein said BLM domain has at least 20% sequence identity to amino acids 95 to 231 of GRB-7.

17. The peptide of claim 10, wherein said BLM domain has at least 30% sequence similarity to amino
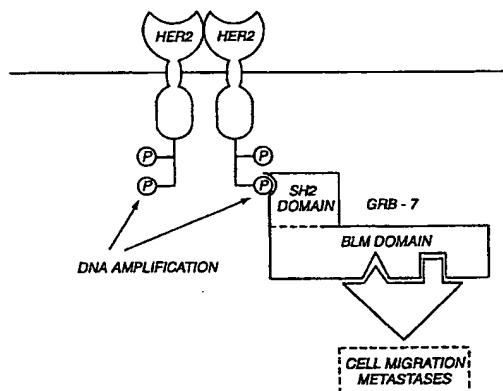15 acids 95 to 428 of GRB-7.

PCT

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2187781

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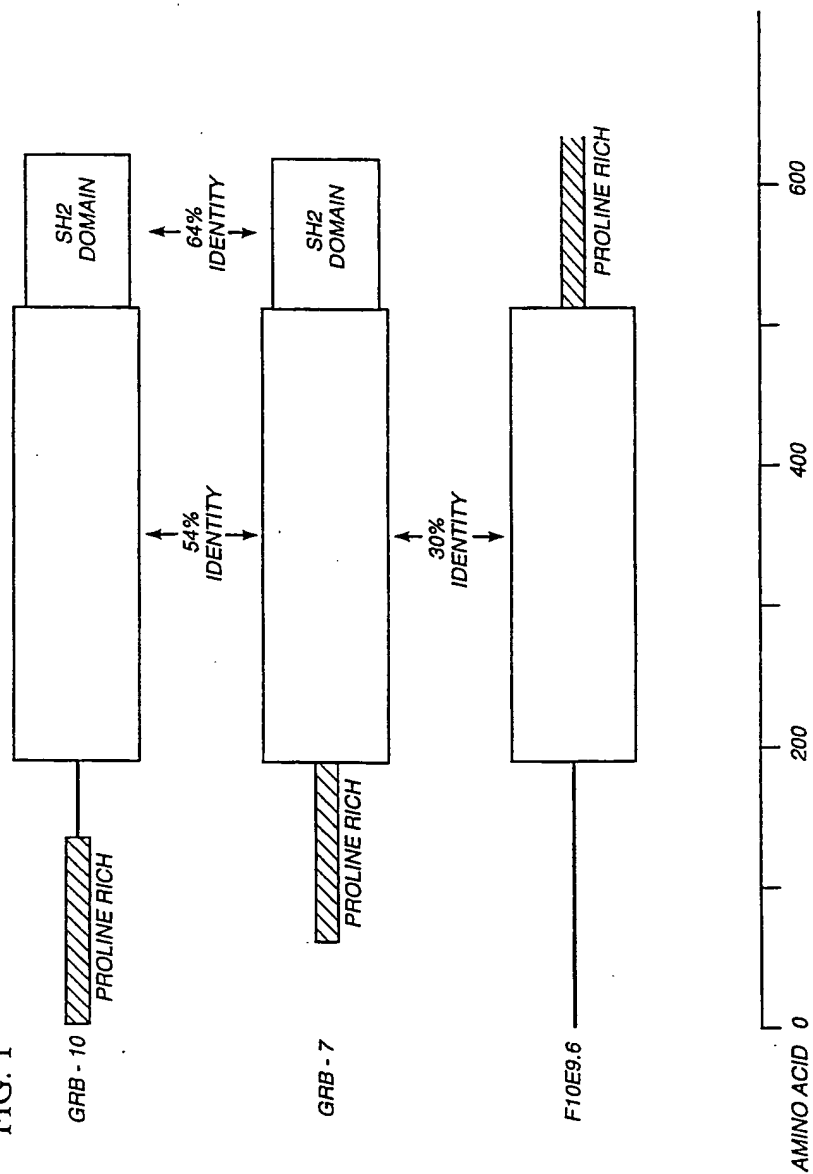   With international search report.

(54) Title: METHODS FOR TREATMENT OR DIAGNOSIS OF DISEASES OR CONDITIONS ASSOCIATED WITH ABNORMAL SIGNAL TRANSDUCTION

(57) Abstract

Methods for diagnosis or treatment of a disease or condition in an organism characterized by an abnormal level of interaction between: (a) a BLM domain and its natural binding partner; or (b) an EGF receptor and an EGF ligand is described. The disease or condition may also be characterized by an abnormality in a signal transduction pathway, wherein the pathway contains a protein with a BLM domain or an EGF receptor. The method includes disrupting or promoting that interaction (or signal) in vivo. The method also involves inhibiting the activity of the complexes formed between the individual molecules. Also, a method for screening for an agent useful for treatment of such a disease or condition by assaying potential agents for the ability to disrupt or promote that interaction is described. The invention also features a peptide comprising, consisting or consisting essentially of a BLM domain or EGF receptor domains 1 or 3.

FIG. 1

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   GRB-7   (95)   pRDssRLc.v  VKVYSEDGac  RsVEVaagaT  ARhVCeMLVq  RaHaLsDESW
   GRB-10  (189)  .mEkIRLRkd  VKVFSEDGts  KvVEIltdmT  ARDLCqLLVy  KsHcVdDnSW
   F10E9.6 (187)  .KEakvtKif  VKfFvEDGea  lqLlIderwT  vaDtlkqLae  KnHialmEdh
   Consensus      --e-------  VK-f-EDG--  --v-i----T  -------L--  k-H-------

   GRB-7   (143)  gLVEsHPyLa  LERgLEDHEf  VVEVqeaWPv  ggDSRFIFRK  NFAKYELFKs
   GRB-10  (238)  tLVEhHPqLg  LERcLEDHEi  VVqVestmP.  .SESKFLFRK  NYAKYEFFKn
   F10E9.6 (236)  cIVEeyPeLy  IkRvyEDHEk  VVEniqmWvq  dSpnKLyFmR  rpdKYaFisr
   Consensus      -lVE--P-L-  l-R--EDHE-  VV--------  -----f-F-k  ---KY-f---

   GRB-7   (193)  PphtLFPEKM  VssCldaqtG  isheDLIQNF  L......Nag  SfPEIQGFLQ
   GRB-10  (286)  Pvn.FFPDqM  VnwCqqsnGG  ..qapVLQNF  L......Nts  ScPEIQGFLQ
   F10E9.6 (286)  PelyLLtpKt  sdhmeipsGd  qwtiDVkQkF  Vseyfhrepv  vpPEmeGFLy
   Consensus      P---lf----  ----------  -----l-Q-F  l---------  --PE--GFL-

   GRB-7   (237)  LRgsGRgSgr  klWKRFFcfL  RRSGLYYSTK  GTSKDPRHLQ  YVADVnESnV
   GRB-10  (327)  VKevGRKS..  ..WKKLYvcL  RRSGLYYSTK  GTSKEPRHLQ  lLADLeESsl
   F10E9.6 (336)  LKsdGRKS..  ..WKKhYfvL  RpSGLYYapK  skkpttKdLt  CLmnLhsnqV
   Consensus      lk--GR-S--  --WKk-y--L  R-SGLYY--K  ------r-L-  -l--l----v

   GRB-7   (287)  YvVtqGRKlY  gmPTDFGfCV  KPNKLRnghK  gL.hIFCsED  EQsRTCWLaA
   GRB-10  (373)  FyLIaGKKqY  naPnEhGmCI  KPNKaKtemK  eL.RLLCAED  EQiRTCWMtA
   F10E9.6 (382)  YtgIgweKkY  ksPTpWcisI  KltaLqmkrs  qFiKyICAED  EmtfkkWLvA
   Consensus      y------K-Y  --P------i  K---------  -l---lC-ED  E-----W--A

   GRB-7   (336)  FRLFKYGvqL  YkNYqqA..Q  sRhLrlsYlg  spPLRSVSDN  tLVAMDFSGH
   GRB-10  (422)  FRLLKYGmlL  YqNYrip..Q  RKgLppPF..  naPMRSVSEN  SLVAMDFSGq
   F10E9.6 (432)  LRIaKnGaeL  leNYerAcqi  RRetlgPass  msaasSstai  SeVphsLShH
   Consensus      fRl-K-G--L  --NY------  -r--------  -----S----  --V---fS--

   GRB-7   (384)  ..........._..aGRVIDNP  rEALSAAMEE  aqAWRkktnh  rLSLpttcs.
   GRB-10  (468)  ..........  ..iGRVIDNP  aEAqSAALEE  ghAWRNgStr  mnilSsSqspl
   F10E9.6 (482)  qrtpsvassi  qlsshmmnNP  thpLSvnV..  ....RNqSpa  sFSVnScqqs
   Consensus      ----------  --------NP  ----S-----  ----R-----  ---l------

   GRB-7   (421)  .gSsLSAaI
   GRB-10  (506)  HPStLnAvI .
   F10E9.6 (526)  HPSrtSAkL
   Consensus      --S---A-i
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FIG. 2

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GRB-7    (2)    ELDLSPTHLSSSPEDVCPTPATP..............PETPPPPDNPPPG
                  |||           ||   ||
GRB-10   (4)    DINSSVESLNSACNMQSDTDTAPLLEDGQHASNQGAASSSRGQPQASPRQ

GRB-7    (38)   DVKRSQPLPIPSSRKLREEEFQATSLPSIPNPFPELCSPPSQKPILGGSS
                  |||  ||     ||   |||  ||||||||
GRB-10   (54)   KMQRSQPVHILRRLQEEDQQLRTASLPAIPNPFPELTGAAPGSPPSVAPS

GRB-7    (88)   GA..............................................
GRB-10   (104)  ..SLPPPPSQPPAKHCGRCEKWIPGENTRGNGKRKIWRWQFPPGFQLSKLTR

GRB-7    (90)   RGLLPRDSSRLC.......................VVKVYSE
                  |||     |                          |||| ||
GRB-10   (154)  PGLWTKTTARFSKKQPKNQCPTDTVNPVARMPTSQMEKLRLRKDVKVFSE

GRB-7    (109)  DGACRSVEVAAGATARHVCEMLVQRAHALSDESWGLVESHPYLALERGLE
                  ||   ||    |||  || |  |   |  |||| ||| || | |||| |
GRB-10   (204)  DGTSKVVEILTDMTARDLCQLLVYKSHCVDDNSWTLVEHHPQLGLERCLE

GRB-7    (159)  DHEFVVEVQEAWPVGGDSRFIFRKNFAKYELFKSPPHTLFPEKMVSSCLD
                  ||| || |         |  |  | |||| |   |||||
GRB-10   (254)  DHEIVVQVESTMP..SESKFLFRKNYAKYEFFKNPVN.FFPDQMVNWCQQ

GRB-7    (209)  AQTGISHEDLIQNFLNAGSFPEIQGFLQLRGSGRGSGRKLWKRFFCFLRR
                    | ||||| | ||||||||   ||| ||    ||        |||
GRB-10   (301)  SNGG..QAQLLQNFLNTSSCPEIQGFLQVKEVGRKS....WKKLYVCLRR

GRB-7    (259)  SGLYYSTKGTSKDPRHLQYVADVNESNVYVVTQGRKLYGMPTDFGFCVKP
                  |||||||||||| |||||  ||   ||       | |    |    |||
GRB-10   (345)  SGLYYSTKGTSKEPRHLQLLADLEESSIFYLIAGKKQYNAPNEHGMCIKP

GRB-7    (309)  NKLRNGHKGLHIFCSEDEQSRTCWLAAFRLFKYGVQLYKNYQQAQSRHLR
                  ||   | | |   |||| ||||   |||| ||||  || || | || | |
GRB-10   (395)  NKAKTEMKELRLLCAEDEQIRTCWMTAFRLLKYGMLLYQNYRIPQRKGLP

GRB-7    (359)  LSYLGSPPLRSVSDNTLVAMDFSGHAGRVIDNPREALSAAMEEAQAWRKK
                    ||||  | |||||| ||||||||  || ||||| ||| |  ||| |||
GRB-10   (445)  PPF..NAPMRSVSENSLVAMDFSGQIGRVIDNPAEAQSAALEEGHAWRNG

GRB-7    (409)  TNHRLSLPTTCS..GSSLSAAIHRTQPWFHGRISREESQRLIGQQGLVDG
                    |  |       |  ||||||| |||| ||||||||| |  |||||||||
GRB-10   (493)  STRMNILSSQSPLHPSTLNAVIHRTQHWFHGRISREESHRIIKQQGLVDG

GRB-7    (457)  VFLVRESQRNPQGFVLSLCHLQKVKHYLILPSEDEGCLYFSMDEGQTRFT
                  || | ||  || ||| ||| |  || |   |   |||  |  ||  |
GRB-10   (543)  LFLLRDSQSNPKAFVLTLCHHQKIKNFQILPCEDDGQTFFTLDDGNTKFS

GRB-7    (507)  DLLQLVEFHQLNRGILPCLLRHCCARVAL
                  || ||| | ||| |||| | ||| |||||
GRB-10.  (593)  DLIQLVDFYQLNKGVLPCKLKHHCIRVAL
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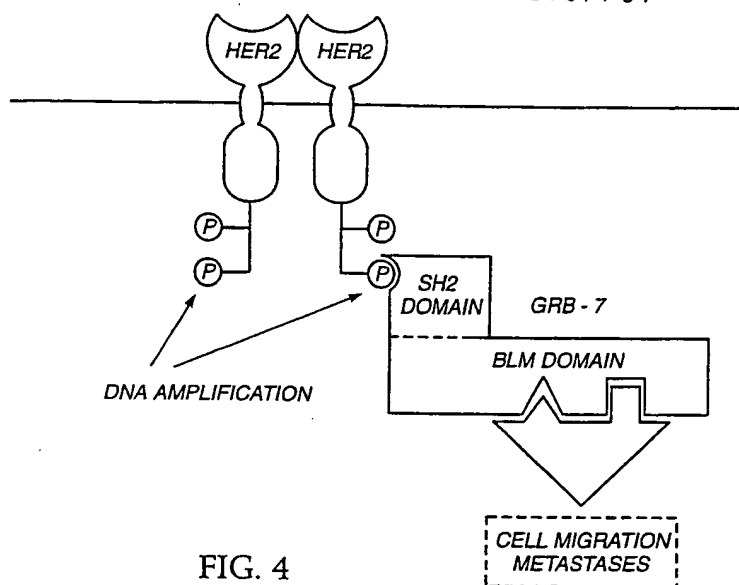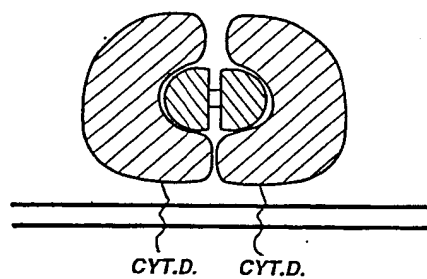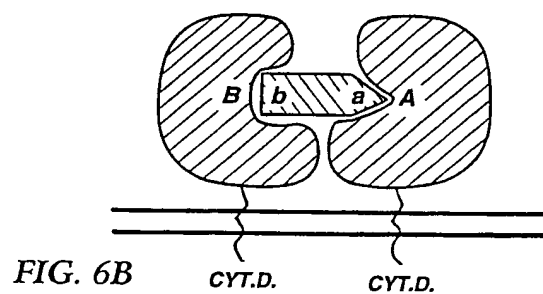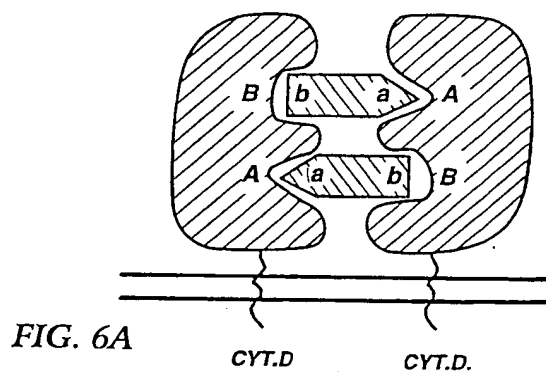
FIG. 3

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FIG. 4

FIG. 5   SUBSTITUTE SHEET (RULE 26)

FIG. 6A

CYT.D. CYT.D.

FIG. 6B

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FIG. 6C